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Roskamp Institute



Therapeutic Target Identification, Validation and Drug Discovery for Traumatic Brain Injury

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Declaration

I hereby declare that the work presented in this thesis is my own, except where stated otherwise. This work has not been submitted for any other degree or professional qualification.

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“Treatment with Nilvadipine Mitigates Inflammatory Pathology and Improves Spatial Memory in Aged hTau mice after Repetitive Mild TBI”

Morin A., Mouzon B., Ferguson S., Paris D., Saltiel N., Lungmus C., Mullan M., Crawford F.

2. Brain Injury 2018; 32(10): 1285-1294 doi: 10.1080/02699052.2018.1486457

“Impact of age on acute post-TBI neuropathology in mice expressing humanized tau: a Chronic Effects of neurotrauma Consortium Study”

Mouzon B., Saltiel N., Ferguson S., Ojo J., Lungmus C., Lynch C., Algamal M., Morin A., Carper B., Bieler G., Mufson E., Stewart W., Mullan M., Crawford F.

3. Frontiers in Aging Neuroscience 217 Dec 22; 9:416. doi: 10.3389/fnagi.2017.00416

“Negative Impact of Female Sex on outcomes from Repetitive mild Traumatic brain Injury in hTau Mice is Age Dependent: A Chronic Effects of Neurotrauma Consortium Study”

Ferguson S., Mouzon B., Lynch C., Lungmus C., Morin A., Crynen G., Carper B., Bieler G., Mufson E., Stewart W., Mullan M., Crawford F.

Abstract

Traumatic Brain Injury (TBI) is a recognized cause of long-term disability worldwide with mild TBI accounting for 80% of all head traumas. Growing evidence links mTBI, and particularly repetitive mTBI (r-mTBI), with long lasting pathological and cognitive deficits that can serve as a risk factor for neurodegenerative disorders such as Alzheimer's Disease, Parkinson's Disease, Chronic Traumatic Encephalopathy and others. So far, there is no FDA-approved treatment to mitigate the consequences of r-mTBI, mainly due to the lack of an effective therapeutic target and a poor translatability of existing preclinical studies, which fail to mimic heterogeneous nature of TBI. In the current thesis, I used a mouse model of r-mTBI which was treated with two different drugs, nilvadipine and anatabine, that have been previously shown to decrease inflammation and neurodegenerative mechanisms and improve cognition. To address the heterogeneous nature of r-mTBI, I used several cohorts of mice which vary in age at injury (young vs old), number of hits (5 vs 24), acute or chronic duration of treatment, and the time of the first treatment intervention post injury (immediate vs delayed). I have found that both nilvadipine and anatabine, in their respective treatment paradigms, improved cognitive deficits, decreased neuroinflammation, and reduced tau pathology. Moreover, nilvadipine was equally effective in both young and old 5-hit r-mTBI mice during the acute treatment. Anatabine was shown to be effective as a delayed treatment starting at 3 months after the last injury in r-mTBI mice with both 5 and 24 hits. We further conducted a phosphoproteome analysis to identify common alterations in response to r-mTBI and tested therapeutics. Despite a high heterogeneity of the phosphoproteome profile between the analyzed cohorts, our data identified several molecules (*ARPP21*, *Syt-1*) which were equally altered in response to treatment in all r-mTBI cohorts and may represent potential therapeutic targets that are effective across different models of r-mTBI. Future studies will focus on the total proteome analysis and a subsequent validation of these potential targets.

Table of contents

Chapter 1 - General Introduction

1.1 Traumatic Brain Injury Epidemiology and Statistics	1
1.2 Classification of Traumatic Brain Injury	3
1.3 Mild TBI and repeated mild TBI in humans	4
1.3.1 Mild TBI Clinical Presentation.....	4
1.3.2 Mild TBI Pathology in humans.....	5
1.3.3 Chronic outcomes of mTBI and related disorders.....	9
1.4 Animal models of mild TBI	12
1.4.1 Review of preclinical injury models.....	12
1.4.2 Mild TBI pathology in animal models.....	15
1.5 Therapeutic approaches for TBI	22
1.5.1 Clinical Trials for TBI.....	22
1.5.2 Preclinical treatment studies for mTBI.....	27
1.5.3 Problems of translation.....	30
1.6 Potential therapeutics developed in The Roskamp Institute	36
1.7 Hypothesis	36
1.8 References	38

Chapter 2 – Investigation of the effect of acute treatment with nilvadipine in the aged hTau mice following r-mTBI

2.1 Introduction	74
2.1.1 Nilvadipine is a potential treatment for mTBI	74
2.1.2 Rationale for using age mice of r-mTBI	76
2.1.3 Hypothesis	78
2.2 Materials and Methods	79
2.2.1 Animals	79
2.2.2 Experimental groups and study design	79
2.2.3 Injury protocol	80
2.2.4 Treatment	81
2.2.5 Motor function assessment	81
2.2.6 Cognitive function assessment	82
2.2.7 Euthanasia	83
2.2.8 Tissue processing and immunohistochemistry	83
2.2.9 Imaging	84
2.2.10 Statistical analysis	85
2.3 Results	87
2.3.1 Behavior assessment	87
2.3.2 Immunohistochemistry	90
2.4 Discussion	95
2.5 References	99

Chapter 3 – Investigation of the effect of treatment with nilvadipine and selective syk inhibitor in the young hTau mice following r-mTBI

3.1 Introduction	106
3.1.1 TBI in the aged and young brain	106
3.1.2 Hypotensive properties of nilvadipine	109
3.1.3 Hypothesis	110
3.1 Methods	111
3.2.1 Animals	111
3.2.2 Experimental groups and study design	111
3.2.3 Injury Protocol	112
3.2.4 Treatment	113
3.2.5 Motor and cognitive functions assessment	113
3.2.6 Tissue collection and processing	114
3.2.7 Immunohistochemistry	114
3.2.8 Fluorescent Staining	114
3.2.9 Immunohistochemical quantification	115
3.2.10 MesoScale Discovery (MSD) Multi-Spot Assay	115
3.2.11 Statistical analysis	116
3.2 Results	117
3.3.1 Motor assessment	117
3.3.2 Memory assessment	118
3.3.3 Immunohistochemistry	121
3.3.4 Inflammatory cytokines	126
3.3.5 Tau phosphorylation	128
3.3.6 Phosphorylated syk	129
3.3 Discussion	131
3.4 References	135

Chapter 4 – Investigation of delayed treatment with anatabine in two different injury paradigms of r-mTBI

4.1. Introduction	140
4.1.1 Anatabine	141
4.1.2 Rationale for using two models of r-mTBI	143
4.1.3 Rationale for using delayed treatment	144
4.1.4 Hypothesis	145
4.1 Materials and Methods	146
4.2.1 Animals	146
4.2.2 Experimental groups and study design	146
4.2.3 Behavior	148
4.2.4 Tissue processing and immunofluorescence	149
4.2.5 Statistical analysis	150
4.2 Results	151
4.3.1 Motor assessment	151
4.3.2 Memory assessment	152
4.3.3 Disinhibition assessment	155
4.3.4 Immunohistochemistry	156
4.3 Discussion	162
4.4 References	167

Chapter 5 – Proteomic approach to the identification of phosphorylated proteins across different models of r-mTBI and treatment paradigms in order to determine common therapeutic targets

5.1. Introduction	177
5.2. Methods	180
5.2.1 Study groups.....	180
5.2.2 Tissue extraction and homogenization.....	180
5.2.3 Sample Preparation.....	181
5.2.4 Protein Digestion.....	182
5.2.5 Peptide Labeling.....	182
5.2.6 Phosphopeptide-enrichment.....	183
5.2.7 LC/MS/MS Analysis.....	184
5.2.8 Peptide and Protein Identification and Quantification.....	184
5.2.9 Data Analysis.....	185
5.3 Results	186
5.3.1 Proteome profiles in different models of r-mTBI.....	186
5.3.2 Phosphoprotein changes across different models.....	190
5.4 Discussion	192
5.5 References	197

Chapter 6 – Summary	201
6.1 Addressing Aim 1	201
6.2 Addressing Aim 2	203
6.3 Addressing Aim 3	205
6.4 Addressing Aim 4	206
6.5 Future Directions	206
6.6 Conclusions	207
6.7 References	208

List of figures and tables

Figure 1. Study Design (Chapter 2).....	80
Figure 2. Behavior evaluation.....	89
Figure 3. Evaluation of the effect of nilvadipine on astrocytes in the aged r-mTBI mice.....	91
Figure 4. Evaluation of the effect of nilvadipine on microglia in the aged r-mTBI mice.....	92
Figure 5. Evaluation of the effect of nilvadipine on p-tau in the aged r-mTBI mice.....	93
Figure 6. Evaluation of the effect of nilvadipine on psyk in the aged r-mTBI mice.....	94
Figure 7. Study Design (Chapter 3).....	112
Figure 8. Evaluation of the effect of nilvadipine or ARC031 on motor performance in the young r-mTBI mice.....	117
Figure 9. Evaluation of learning and memory in the young r-mTBI mice.....	119
Figure 10. Age-dependent behavior deficits in sham-vehicle mice.....	121
Figure 11. Evaluation of the effect of nilvadipine or ARC031 on astrocytes	123
Figure 12 Immunofluorescent staining of GFAP in cortex in the young r-mTBI mice.....	124
Figure 13. Immunofluorescence of GFAP in cortex, corpus callosum and hippocampus in the young (3 months) and aged (24 months) mice with r-mTBI.....	125
Figure 14. Evaluation of the effect of nilvadipine on microglia in the young r-mTBI mice....	126
Figure 15. Quantitative analysis of proinflammatory cytokines in cortex of young and aged hTau mice.....	127
Figure 16. Quantitative analysis of p-tau, total tau and p/tau/total tau in cortex of young and aged hTau mice.....	128
Figure 17. Evaluation of the effect of nilvadipine or ARC031 on psyk, GFAP and Iba1 in corpus callosum.....	129
Figure 18. Proposed mechanism of action of anatabine.....	143
Figure 19. Study Design (Chapter 4).....	148
Figure 20. Evaluation of the effect of anatabine on motor performance.....	151
Figure 21. Evaluation of learning and memory in cr-mTBI and 5r-mTBI mice.....	153
Figure 22. Probe trial in the cr-mTBI and 5r-mTBI mice.....	154
Figure 23. Evaluation of disinhibition in the cr-mTBI and 5r-mTBI mice.....	155

Figure 24. Evaluation of the effect of anatabine on microglia in the cr-mTBI and 5r-mTBI mice.....	157
Figure 25. Immunofluorescent analysis of GFAP and NFkB in the cr-mTBI and 5r-mTBI mice.....	158
Figure 26. Representative images of NFkB in cortex and CA1 area of hippocampus in 5r-mTBI and cr-mTBI mice.....	160
Figure 27. Evaluation of the effect of anatabine on RZ3 in cortex in the cr-mTBI and 5r-mTBI mice.....	161
Figure 28. Protein-protein interactions in the cr-mTBI/A cohort.....	187
Figure 29. Protein-protein interactions in the 5r-mTBI/A cohort.....	188
Figure 30. Protein-protein interactions in the 5r-mTBI/N cohort.....	189
Figure 31. Venn diagrams of the dysregulated phosphoproteins in response to TBI-V and TBI-T.....	190
Figure 32. Heatmap of selected phosphoproteins.....	191
Figure 33. Variability of the analysis methods of the phosphoproteome data.....	192
Table 1. Summary of experimental models of mTBI in rodents (Chapter 1).....	14
Table 2. Distribution of mice in the groups (Chapter 4).....	147
Table 3. Age of mice across the major intervention steps (Chapter 4).....	166
Table 4. Abbreviations of the study cohort and treatment groups (Chapter 5).....	180
Table 5. Time points (Chapter 5).....	181

List of abbreviations

AD – Alzheimer's Disease

AED – Animal Equivalent Dose

ALS – Amyotrophic Lateral Sclerosis

ANOVA – Analysis of Variance

APP – Amyloid Precursor Protein

ARTAG – Aging-Related Tau Astroglipathy

ARRP21 - cAMP-Regulated Phosphoprotein 21

BBB – Blood Brain Barrier

BCA – Bicinchoninic Acid

BM – Barnes Maze

cAChR – Nicotinic Acetylcholine Receptor

CBF – Cerebral Blood Flow

CCB – Calcium Channel Blocker

CCI – Controlled Cortical Impact

CCL-2 – C-C Motif Chemokine Ligand 2

CHI – Closed Head Injury

CHIMERA – Closed Head Impact Model of Engineered Rotational Acceleration

CRASH – Corticosteroid Randomization After Significant Head injury

CT – Computer Tomography

CTE – Chronic Traumatic Encephalopathy

CXCR – CXC Chemokine Receptor

DAB - 3,3'-Diaminobenzidine

DAI – Diffuse Axonal Injury

DAMP – Damage Associated Molecular Patterns

DHP – Dihydropyridine

DRS – Disability Rating Scale

DTI – Diffuse Tensor Imaging

EDTA – Ethylenediaminetetraacetic acid

EPM – Elevated Plus Maze

EPO – Erythropoietin

FDA – Food and Drug Administration

FPI – Fluid Percussion Injury

FTLD – Frontotemporal Lobar Degeneration

GCS – Glasgow Coma Scale

GFAP – Glial Fibrillary Acidic Protein

GOS – Glasgow Outcome Scale

HSP – Heat Shock Protein

HRP-3 - Hepatoma-Derived Growth Factor-Related Protein 3

IACUC – Institutional Animal Care and Use Committee

Iba1 – Ionized Calcium Binding Adaptor Molecule 1

ICAM – Intracellular Adhesion Molecule

ICP – Intracranial Pressure

IHC – Immunohistochemistry

IL – Interleukin

i.m. - Intramuscular

iNOS – inducible Nitric Oxide Synthase

i.p. – Intraperitoneal

i.v. – Intravenous

KCGRO – Keratinocyte chemoattractant/human growth-regulated oncogene

LC/MS – Liquid Chromatography – Mass Spectrometry

LFP – Lateral Fluid Percussion

LOC – Loss of Consciousness

LXR – Liver X Receptor

MAPT – Microtubule Associated Protein Tau

MANOVA – Repeated-measure Analysis of Variance

MCI – Mild Cognitive Impairment

MMP-9 – Matrix Metalloproteinase 9

MMSE – Mini Mental State Examination

MPER – Mammalian Protein Extraction Reagent

MRI – Magnetic Resonance Imaging

MSCs – Mesenchymal Stem Cells

MSD – MesoScale Discovery

mTBI – mild Traumatic Brain Injury

NCS – Minimally Conscious State

NF – Neurofilament

NFASC – Neurofascin

NFL – National Football League

NFTs – Neurofibrillary Tangles

NPV – Negative Predictive Value

NSCs – Neural Stem Cells

NSE – Neuron Specific Enolase

NMDA – N-Methyl-D-Aspartic acid

OBTT – Operation Brain Trauma Therapy

OEF – Operation Enduring Freedom

OIF – Operation Iraqi Freedom

OND – Operation New Dawn

PBS – Phosphate-buffered Saline

PEG – Polyethylene glycol

PET – Positron Emission Tomography

PFA – Paraformaldehyde

PHF – Paired Helical Filaments

PSMs – Peptide Spectral Matches

PTA – Post-traumatic amnesia

RI – Roskamp Institute

r-mTBI – repetitive mild Traumatic Brain Injury

ROI – Region of Interest

SAP – Serum Albumin Protein

Syk – Spleen Tyrosine Kinase

Syt-1 – Synaptotagmin 1

TBI – Traumatic Brian Injury

TCEP - Tris(2-carboxyethyl) Phosphine

TEAB – Triethylammonium Bicarbonate Buffer

TMT – Tandem Mass Tag

TNF α – Tumor Necrosis Factor alpha

UCHL-1 – Ubiquitin C-Terminal Hydrolase 1

VEGF – Vascular Endothelial Growth Factor

VG – Vegetative State

VSP13C - Vacuolar Protein Sorting-Associated Protein 13C

Chapter 1 – General Introduction

1.1 Traumatic Brain Injury Epidemiology and Statistics

Globally, 61 million individuals are estimated to sustain Traumatic Brain Injury (TBI) each year (Dewan et al. 2019). In the United Kingdom (UK) alone, TBI-related hospital visits account for 1.4 million cases annually (Lawrence et al. 2016), whereas in the United States (US) TBI affects over 2.5 million people per year and accounts for more than 13 million cases of long-term disability to date (Taylor et al. 2017). The highest incidence of TBI per 100,000 people is recorded in North America (1299 cases) and Europe (1012) (Dewan et al. 2019). These data, however, underestimate the real number of TBI cases because many victims do not seek medical attention, especially in cases of mild forms of TBI (mTBI) when no obvious symptoms are present during the acute and subacute period post injury. For instance, all TBI-related hospital visits between 2014 and 2015 in England and Wales were dominated by mild injuries (68%) while more severe forms of TBI accounted for the remainder (Lawrence et al. 2016). Worldwide, according to the meta-analysis of 121 incidence studies between 1980 and 2000, 42 million people sustain mTBI occupying the majority of all TBI cases (Cassidy et al. 2004). According to the Centers for Disease Control (CDC), the rates of TBI-related Emergency Department (ED) visits increased from 521.0 to 823.7 per 100,000 in the decade between 2001 and 2010 (CDC. Injury prevention & control: traumatic brain injury & concussion <https://www.cdc.gov/traumaticbraininjury/data/rates.html>). Meanwhile, the rates of deaths caused by TBI recorded for the same period decreased from 18.5 to 17.1 per 100,000, likely in part to an increased awareness and medical advancements. Worldwide, TBI is most prevalent in infants (0-4 years), younger adults (15-24 years) and the elderly (> 65 years) (Sarmiento et al. 2019; Centers for Disease Control and Prevention 2011; Flanagan et al. 2006; Nguyen et al.

2016). The most common causes of acquired brain injury include sport-related injuries, motor vehicle accidents, falls and assaults (Rutland-Brown et al. 2006; Lajoie and Gallagher 2004). In the US, the highest incidence of TBI and repetitive TBI is recorded in professional athletes. In the years 2001-2012, sport-related injuries contributed to over 3 million ED visits (Coronado et al. 2015). The number of ED visits was increased for both males and females, with males having twice higher rates of TBI incidence each year. In the survey conducted by Coronado et al., around 90% of all TBI patients were released within days, indicating that milder forms of TBI were the most prevalent (Coronado et al. 2015). Military personnel represent another population group that is associated with high incidence of TBI worldwide. In the US, The Department of Defense (DoD) reported more than 380,000 of the US service members diagnosed with TBI between years 2000 and 2018 (Defense and Veterans Brain Injury Center 2018). Among them, nearly 316,000 (82.3%) individuals had mild form of TBI as assessed by GCS (12-15). In a recent study that evaluated the incidence of TBI in the service members of the Iraq and Afghanistan conflicts (VA TBI Veterans Health Registry 2001-2013), mTBI constituted 87.3% of all brain traumas with blast waves (33.1%) and objects hitting the head (31.7%) being among the most reported reasons (Lindquist et al. 2017). TBI has also been recognized as a signature wound of Operation Enduring Freedom (OEF), Operation Iraqi Freedom (OIF) and Operation New Dawn (OND) where out of 185,437 veterans, who completed the survey, 78% reported exposure to a traumatic event associated with alteration of consciousness. Similarly, 81% of these veterans experienced mTBI with 77% arising from blast injuries (Whiteneck et al. 2015). In the UK armed forces, between 2003 and 2011, 19% of all casualties in Iraq and Afghanistan wars suffered a TBI (Hawley et al. 2015).

1.2 Classification of Traumatic Brain Injury

TBI describes a spectrum of head traumas that can be classified into several types depending on the severity, mechanism, location on the head and other factors (Saatman et al. 2008). The most common tool for classification is the Glasgow Coma Scale (GCS), which is used for clinical diagnoses (Green et al. 2017). It is based on the acute neurological assessment that measures eye, verbal and motor responses. Each parameter is scored individually according to its severity (1-4, 1-5 and 1-6 respectively) with the most severe presentation in each category (e.g. no eye opening, no sounds or no response respectively) having the lowest score of 1; these three scores are then combined in a 3-15 point scale. The 15-point GCS system classifies mild (13-15), moderate (9-12) and severe (3-8) TBI (Johnson et al. 2017). Moderate and severe head injuries are associated with higher hospitalization rates, gross brain pathology and increased mortality. These usually result from a penetrative focal injury that involves skull fracture and brain tissue damage. Such injuries often arise from assaults or accidents. Conversely, mTBI does not lead to gross pathology or strong neurological dysfunctions at the acute hospitalization period. Mild TBI patients may experience short loss of consciousness (LOC) <30 min and brief post-traumatic amnesia (PTA) <24h (Carroll et al. 2004; Eme 2017; Pavlovic et al. 2019). Such injuries include non-penetrative blunt impacts that result in diffuse cellular injury rather than focal tissue loss. Most mTBI result from concussions, falls, blast waves and mild accidents. Assessment by GCS, LOC and PTA is, however, being criticized by the scientific community as insufficient to accurately determine the severity of an injury (Braine and Cook 2017). To overcome this uncertainty, additional tools, including imaging techniques, have been used to differentiate mTBI pathology. Traditionally, computer tomography (CT) scan could not diagnose mTBI due to its low sensitivity (Eme 2017). However, emerging advances in functional

magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) provide the ability to distinguish subtle neuropathological processes such as axonal and vascular injury (Pavlovic et al. 2019).

1.3 Mild TBI and repeated mild TBI in humans.

1.3.1 Mild TBI Clinical Presentation

The immediate response to mTBI includes headaches, dizziness, fatigue and sleep disturbances, and they usually resolve within several days or weeks (more rarely, within couple months) (Katz et al., 2015; Prince and Bruhns 2017). If unresolved, these symptoms may evolve into prolonged neurological problems lasting for months and years post-trauma. For example, memory problems are among the most common deficits reported after mTBI in both acute and chronic periods. Within the first 24 hours post mTBI, patients may have impaired consolidation processes leading to difficulties in memorizing new information, known as post-traumatic amnesia (PTA) (Flynn 2010). Later, within months after mTBI, if memory problems are sustained, they are expressed as difficulties in memory retrieval, impaired working memory and slow speed of processing. However, a comprehensive analysis of neurological deficits should include combinatory and not individual effects of lasting symptoms. For example, mTBI-induced sleep disturbances and insomnia are strongly correlated with memory deficits (Mantua et al. 2017). Adolescents with sport-related concussions report sleep disturbances and demonstrate impaired verbal and visual memory compared to patients who recover regular sleep behavior (Beebe et al. 2018; Kostyun et al. 2015).

Early studies in boxers who sustained multiple concussions throughout their career reported balance problems, memory deficits, personality change, aggressive behavior, mood disorder, depression, suicidal behavior and other neuropsychiatric impairments (Martland 1928; Millspaugh 1937; Critchley 1957). Such clinical presentation of chronic consequences of r-mTBI was referred as ‘punch drunk’ syndrome. Later, similar behavioral outcomes were recorded in former National Football League (NFL) players (“American Football”) who sustained repetitive concussions throughout their career in sport. Omalu and colleagues first described this condition as Chronic Traumatic Encephalopathy (CTE), where former football players experienced chronic depression, suicide attempts, paranoia, insomnia and mood changes at up to 12 years after their retirement (Omalu et al. 2005, 2006, 2010). A more recent review summarized 51 cases of CTE among which 46 occurred in boxers and football players (McKee et al. 2009). Common clinical symptoms included irritability, memory loss, aggressive behavior, gait abnormalities, slurred speech and Parkinsonism. Nearly 50% of described individuals were symptomatic within 4 years after they stopped playing. In recent years, CTE has become a subject of intense media scrutiny raising legal and societal implications (Korngold et al. 2013). Despite increased attention to, and recognition of, the chronic effects and negative sequelae of mTBI, CTE has temporarily subverted TBI field into the belief that all mTBI are equivalent to CTE. Thus far, CTE has been assessed in former athletes with the history of r-mTBI, however, whether sport-related concussions are the definitive cause of CTE remains unclear.

1.3.2 Mild TBI Pathology in humans

Clinical symptoms are essential to distinguishing mTBI from more severe forms of injury at the acute phase, however, they do not provide a comprehensive picture of injury-induced brain pathophysiology. Neuropathological changes in the mTBI brain are responsible for long-lasting

cognitive deteriorations and thus require special attention. There are 3 ways to measure cellular and molecular changes in the brain: live imaging, biomarkers and post-mortem analysis.

Live imaging uses multiple techniques such as CT, MRI, DTI and positron emission tomography (PET) that are able to capture certain morphological and physiological changes, depending on the sensitivity of the technique employed. Due to the mild nature of concussions, most live-imaging techniques fail to detect brain microscopic abnormalities after mTBI (Bigler 2015). For instance, CT and MRI can only detect gross pathology such as skull fracture, bleeding, and edema, none of which are typically present in mTBI (Bigler 2015). Conversely, diffuse imaging techniques such as DTI have higher sensitivity and are able to detect changes in large fiber tracts in white matter such as diffuse axonal injury (DAI) (Bigler 2015; Toth 2015). Although DTI limitations include low specificity to injury-induced vs nonpathological changes of cell integrity, recent advancements have made DTI an attractive technique for mTBI diagnosis. Nevertheless, structural imaging alone does not provide enough information about mTBI pathology (Shin et al. 2017). Instead, functional imaging techniques which measure metabolic changes are more advantageous. For instance, PET imaging can measure glucose uptake which is known to be altered after mTBI. Byrnes and colleagues summarized clinical PET studies for mTBI in their review and highlighted important temporal changes in glucose levels (Byrnes et al. 2014). Several studies confirmed glucose uptake reduction in different regions at both acute and chronic time points after mTBI. For example, veterans with blast-induced concussions showed a decrease of glucose metabolism at 2 years post-TBI in regions varying from frontal and temporal cortex to brainstem and cerebellum (Mendez et al. 2013; Petrie et al. 2014). These findings were confirmed by PET imaging in animal studies where rats were exposed to experimental mild TBI (Selwyn et al. 2013, 2015). Functional MRI (fMRI) is

another imaging approach with high enough sensitivity to detect network connectivity changes in the brain. fMRI scans showed reduced functional connectivity between different brain regions at 90 days post-mTBI in veterans, and decreased interhemispheric connectivity at 10 days post-TBI in athletes (Han et al. 2014; Slobounov et al. 2011).

Peripheral **biomarkers** have proven to be another promising tool to determine TBI occurrence and severity and propose an ongoing pathology. Brain injury leads to the disruption of blood-brain barrier (BBB) and astrocytic/neuronal integrity, which trigger the release of intracellular molecules into the blood stream. Several neuron-specific proteins, such as UCHL1 (Ubiquitin C-Terminal Hydrolase 1), NSE (neuron-specific enolase), NF (neurofilament), and tau, have been recorded to enter the circulation if the BBB integrity is compromised (Kim et al. 2018; Papa et al. 2015; Zetterberg et al. 2013). However, few candidate biomarkers have demonstrated the ability to differentiate mTBI from more severe injuries. For example, elevated levels of phosphorylated tau (p-tau), total tau (t-tau) and the p-tau/t-tau ratio have been found in the plasma of patients with acute and chronic TBI across different severities (Rubenstein et al. 2017). In fact, in this study, the concentration of plasma tau was associated with TBI severity, allowing differentiation of mTBI from more severe injuries. Conversely, some clinical studies suggest that increased tau is only seen in moderate and severe TBI (Lippa et al. 2019). Further, in the study of American football and ice hockey players, plasma tau in athletes with r-mTBI was not increased at 6 days post-injury suggesting specificity of tau as a *chronic* biomarker (Wallace et al. 2018). Another biomarker, neuronal cytoplasmic protein UCHL1, plays an important role in removing abnormal proteins through the ubiquitin-proteasome pathway and autophagy (Liu et al. 2019). Increased levels of UCHL1 were found in the serum of mild and moderate TBI cases within one hour post-injury, and was correlated with CT signal and GCS score (Papa et al. 2012;

Ramezani et al. 2018). Neurofilaments, which are found in axons, have also been found in the serum of mTBI patients within 1 hour and 3 days after the injury corroborated by a negative CT signal, confirming the inability of CT to differentiate between mTBI and controls (Gatson et al. 2014).

In addition to neuronal biomarkers, astrocyte-derived Glial Fibrillary Acidic Protein (GFAP) has also been detected to be elevated for up to 90 days post injury in the blood of mTBI patients (Bogoslovsky et al. 2017). In fact, a combination of GFAP and UCHL1 has been recently approved by the Food and Drug Administration (FDA) as the first biomarker for acute-phase TBI (Bazarian et al. 2018). Studies showed that serum concentrations of GFAP and UCHL1 had a high sensitivity and Negative Predictive Value (NPV) for the detection of intracranial injuries on CT scans, thus allowing avoidance of unnecessary CT imaging for patients with mTBI with GCS 14-15 (Bazarian et al. 2018; Posti et al. 2016). Measurement of GFAP and UCHL1 however provides only an exclusionary biomarker for mTBI, not a diagnostic biomarker. Since very few mTBI victims seek medical attention at acute times, there remains a need for diagnostic biomarkers of mTBI that persist at a chronic period post injury. Several studies of military personnel with a history of mTBI also showed elevated levels of inflammatory cytokines IL-6, IL-10 and TNF α in plasma at 3 - 18 months post injury, suggesting an ongoing inflammation in a concussed brain (Kanefsky et al. 2019; Gill et al. 2018). The use of biomarkers helps to detect distinct pathophysiology after mTBI associated with BBB disruption and neuronal damage, however, they do not allow identification of brain-specific mechanisms that are not associated with BBB damage and resulting leakage into the periphery.

Post-mortem analysis allows for the identification of macroscopic and microscopic changes in different brain regions and thus provides a comprehensive picture of mTBI pathology.

Unfortunately, pathological studies of acute mTBI are rare and usually include more severe injuries. Of the few cases available of early post injury examination of mTBI brains, some reveal normal brain appearance without gross morphological changes after a concussion (Bigler 2004; Blumbergs et al. 1994). On the microscopic level, one study of 5 mTBI victims, who died within 2-99 days post injury from other causes, found consistent axonal injury in the white matter hemispheres, corpus callosum and fornices, which are involved in memory, as assessed by amyloid precursor protein (APP) staining (Blumbergs et al. 1994). Another study of mTBI brains analyzed at couple days to several weeks post-injury demonstrated axonal injury and microglial clustering in the perivascular regions (Oppenheimer 1968). In young (14-28 years old) athletes across different sports who died within 6 months after a reported mTBI, pathological examination showed axonal injury in white matter, perivascular microgliosis and astrogliosis, and focal accumulations of p-tau (PHF) (McKee et al. 2014). To our knowledge, these are the only studies that performed acute pathological analysis after mTBI, which is likely due to very few cases of immediate death after mild head injury. Indeed, most autopsy examinations are performed at chronic timepoints after mTBI, especially in cases when multiple concussions are associated with neurodegenerative disorders.

1.3.3 Chronic Outcomes of mTBI and related disorders

The first evidence of the chronic effects of r-mTBI was recorded by Dr. Martland when he described ‘punch drunk’ syndrome in boxers with the history of multiple concussions who developed long-lasting motor, cognitive, and behavior abnormalities (Martland 1928). In 1937, Millspaugh introduced the term ‘dementia pugilistica’ by commenting on Martland’s findings, suggesting the possibility for microscopic changes in these brains, which were later confirmed by Corsellis (Millspaugh 1937, Corsellis et al., 1973). Next, Dr. Bennet Omalu described CTE

when he analyzed the brains of former NFL players who died at the age of 45 or 50, 12 years after their retirement from professional football that involved multiple concussions (Omalu et al. 2005, 2006). Post-mortem analysis revealed the presence of neurofibrillary tangles (NFTs) in the neocortical areas of both cases, whereas the first case (Omalu et al. 2005) was also characterized by the presence of diffuse amyloid plaques. Later, Dr. Omalu presented the third case of CTE in a former NFL player who died at the age of 44, 11 years after his retirement from professional sport, and exhibited NFTs but no amyloidopathy (Omalu et al. 2010). Medical records from all 3 cases reported neuropsychiatric history and major depressive disorders between the end of their career and death. Additional studies later confirmed that CTE patients share common symptoms such as irritability, memory loss, confusion, mood disturbances, gait abnormalities and speech problems (McKee et al. 2009).

Neuropathologically, CTE was characterized by atrophy of cerebral hemispheres, thalamus, brainstem and medial temporal lobe, dilation of ventricles and fenestration of the cavum septum pellucidum (McKee et al. 2009). On the microscopic level, multiple autopsy analyses demonstrated presence of tau pathology, neurofibrillary threads, endoplasmic reticulum stress, inflammation and cerebrovascular damage (Lucke-Wold et al. 2015; McKee et al. 2009; Omalu et al. 2011). A broad spectrum of pathophysiological outcomes in CTE cases and its overlap with other neurodegenerative disorders undermines the diagnostic accuracy. A consensus panel comprised of independent neuropathologists defined the criteria for CTE diagnosis based on 25 tauopathy cases and included abnormal accumulation of hyperphosphorylated tau around blood vessels and in the depth of cortical sulci as the main symptoms (McKee et al. 2016). However, an early diagnosis of CTE is based solely on clinical examination by assessing cognitive abnormalities associated with mood and behavior dysfunctions (Montenigro et al.

2014). Although advancements have been made in identifying CTE, the idea of defining CTE as a distinct disease remains debatable. A recent study showed that not all athletes who received repetitive concussions develop CTE, despite a positive neurological assessment (Lee et al. 2019). Lee and colleagues suggest that CTE often develops as a co-morbidity to other diseases and not as a primary dementia. One study also presented a case of CTE-like pathology in a 45-year old man with 7-year history of ALS and no known records of TBI (Gao 2017). Postmortem examination demonstrated NFTs in the superficial layers of neocortex, areas surrounding blood vessels and in the depth of sulci in the frontal and temporal lobes as well as glial tau tangles and diffuse amyloid plaques.

In brains with chronic TBI, astroglia have been shown to surround tau oligomers suggesting a strong link between inflammation and tau pathology. Hyperphosphorylated astrocytic tau impairs normal astroglial functions, leading to BBB disruption, decreased glutamate re-uptake and increased inflammation (Kahlson and Colodner 2015). A spectrum of astroglial tau pathology in neurodegenerative disorders has been named Aging-Related Tau Astroglipathy (ARTAG) and is mainly detected in the elderly (Kovacs et al. 2016). ARTAG astrocytes are usually thorn-shaped and have fine granular immunoreactivity in the astrocytic processes, which distinguish them from the primary tauopathies. Although more research is necessary, ARTAG has been found in 455 of 687 cases across different neurodegenerative disorders including frontotemporal lobar degeneration (FTLD), Pick's disease, Lewy body disease, AD and others (Kovacs et al. 2018). To date, there are no studies linking ARTAG and mTBI, however neuropathological similarities between the two warrant further investigation.

1.4 Animal models of mild TBI

1.4.1 Review of preclinical injury models

Preclinical studies are crucial in understanding cellular aspects of mTBI neuropathology, assessing potential therapeutic targets and testing prospective drugs prior to clinical trials. Numerous animal models of brain injuries have been developed in flies, fish, pigs, rodents, ferrets, sheep and non-human primates (Bolouri and Zetterberg 2015; Sorby-Adams et al., 2018). Although large animals, such as pigs, sheep and non-human primates, may seem to be a better model because they have gyrencephalic brains (as do humans), most mTBI (and r-mTBI) studies use rodents due to their availability, low cost, high reproducibility and easy manipulation. These models have been proven to closely replicate many aspects of human mTBI pathobiology such as axonal injury, inflammation, tauopathy, oxidative stress, BBB disruption and cognitive deficits such as memory loss, anxiety, social interaction, stress and motor impairments (Bolouri and Zetterberg 2015).

Table 1 summarizes rodent models of mTBI that are commonly used in pre-clinical research (for review see Bolouri and Zetterberg 2015 and Bolton-Hall et al. 2019). All these models can be divided into closed-head or open-skull injuries. Open-skull injuries include controlled cortical impact (CCI) and fluid percussion injury (FPI), both of which require craniectomy prior to the mechanical impact (Morganti-Kossmann et al. 2010). CCI uses an impactor device with pre-determined velocity and depth of penetration. It results in a focal injury, usually affecting a single hemisphere and causing loss of tissue (Romine et al. 2014). Although multiple studies refer to their CCI models as mTBI, sham mice for such models are themselves experiencing a mild TBI through the craniectomy. Therefore, mice receiving a CCI injury are more likely to replicate moderate and severe TBI and thus such data should be viewed

in that regard (Lagraoui et al. 2012, Ferguson et al. *pers comm*). A benefit of using the FPI model is its ability to cause diffuse injury by applying a high-pressure pulse of fluid onto the intact brain through the craniectomy window. FPI is a widely-accepted model for mTBI that does not cause tissue loss or gross pathological symptoms, however, again, craniectomy by itself induces injury-like pathology such as neuroinflammation and cerebrovascular damage in sham animals confounding data interpretation (Cole et al. 2011; Sargolzaei et al. 2018). Hence, alternative models using closed head injury (CHI) are more relevant for mimicking concussions in animals. One such model is a Marmarou's weight drop model of TBI that uses a free-falling weight on the closed head (Marmarou et al. 1994). It triggers the anticipated mTBI pathology of axonal injury, inflammation and long-lasting cognitive deficits (Zohar et al. 2003; Tweedie et al. 2016; Milman et al. 2005). However, due to poor control of the impact driven solely by gravity force, the results can vary from study to study. Another limitation of the previously described models is the lack of information on how head movement contributes to a concussion. In humans, a vast number of injuries involve head rotation due to acceleration and deceleration. This rotation results in the movement of the brain inside the skull and consequently produces two impact areas. However, none of the previously described models consider free head movement. The most recent Closed Head Impact Model of Engineered Rotational Acceleration (CHIMERA) is designed to address this limitation and account for biomechanical aspects of head impact (Namjoshi et al. 2014). This model is advantageous in mimicking repeated injuries which replicate human r-mTBI pathology such as diffuse axonal injury, excessive microgliosis and astrogliosis, and tau phosphorylation both acutely and chronically (Namjoshi et al. 2014; Cheng et al. 2019). CHIMERA mice also exhibit neurological, motor and cognitive deficits with anxiety-like behavior.

Injury model	Benefits	Drawbacks	References
CCI	High reproducibility of the focal impact; controlled impact variables.	Craniectomy induces mTBI-like pathology in the sham; focal impact; fixed head, no rotation; craniectomy position may cause variable outcomes between laboratories.	(Onyszchuk et al. 2008; Romine et al.2014)
FPI/LFP	High reproducibility of the impact; controlled impact variables; diffuse impact.	Craniectomy induces mTBI-like pathology in the sham; fixed head, no rotation; craniectomy position may cause variable outcomes between laboratories; focal cell loss is sometimes present.	(Alder et al. 2011; Carbonell et al. 2009; Lyeth 2016; Thompson et al. 2005)
Weight Drop	Closed head injury; no craniectomy induced pathology; possibility to induce free movement of the head and body upon the impact (including falls); diffuse impact.	Low reproducibility due to little control over impact variables (i.e. gravity force); inability to visualize anatomical landmarks of impact; poor comparability between the studies.	(Abd-Elfattah Foda and Marmarou 1994; Zohar et al. 2003; Marmarou et al. 1994)
Blast Injury	Closed head injury; no craniectomy induced pathology in sham animals; diffuse impact; may trigger head movement upon the blast impact; high relevance to military blast injuries.	High variability between and within models due to: different blast sources, variability in the distance between the blast source and the head, little control over the blast forces propagation through the air	(Perez-Polo et al. 2015; Mendez et al. 2013; Wang et al. 2016)
CHIMERA	Closed head injury; no craniectomy induced pathology in sham animals; high reproducibility and comparability between the studies; rotational movement of the head	Focal injury; inability to visualize anatomical landmarks for the impact; this model introduces a new variable - the injury tolerance depending on the angular plane.	(Cheng et al. 2018, 2019; Namjoshi et al. 2014, 2017)
Closed Head CCI	Closed head injury; no craniectomy induced pathology in sham animals; high reproducibility and comparability between the studies.	Fixed head, no rotation; inability to visualize anatomical landmarks for the impact.	(Mouzon et al. 2012)

Table 1. Summary of experimental models of mTBI in rodents

Finally, animal models have also been developed which replicate concussions in the military personnel resulted from explosions (Mendez et al. 2013; Perez-Polo et al. 2015). These experimental models use blast waves in the proximity of an animal to cause a blast decompressive injury. This type of injury does not produce skull fracture or gross pathology and replicates a typical response to mTBI. Blast injury in mice induces gliosis, axonal injury, BBB disruption and severity-dependent behavior deficits (Wang et al. 2016). However, limitations in measuring remote blasts and high variability of the distance from the blast source across different studies make direct comparison difficult. In general, clinically relevant models of mild TBI demand the following characteristics: 1) closed head, 2) mild level of impact, 3) low to none mortality rate and 4) ability to cause mTBI pathology without gross symptoms (Bolouri and Zetterberg 2015).

1.4.2 Mild TBI pathology in animal models

Axonal Injury

Mild TBI is a heterogeneous condition that triggers a series of pathological events lasting from seconds and minutes to days, months and even years. The immediate response of brain cells to the mechanical impact is called primary injury. At this stage, mechanical stress affects neurons and neuronal structures leading to either cell death or cell damage depending on the proximity of cells to the impact areas, propagation of stress, and individual cell characteristics. The key elements of primary injury are shear and stretch forces that result from rotational

acceleration/deceleration and stress propagation in the brain (Siedler et al. 2014). Axonal structures have been shown to be the most vulnerable structures to such forces which result in axonal injury. Most animal models of mTBI closely replicate human axonal pathology, which is identified by the presence of bulbs and swellings inside the axons. These morphological changes result from the microtubules mislocation and accumulation of amyloid precursor protein (APP) (Tang-Schomer et al. 2012; DiLeonardi et al. 2009). APP is a transmembrane protein that has been implicated in axonal transport by anchoring cargo proteins and vesicles to the motor protein associated with microtubules (Muresan et al. 2009). Historically, APP has been used as a marker for axonal injuries and has been demonstrated in numerous animal models of mTBI, particularly at acute timepoints. Another common marker of axonal injuries is neurofilament which in *in vitro* models of mild stretch injury show a strong immunoreactivity forming ring-like structures with axonal swellings (Chung et al. 2005; Dunn-Meynell and Levin 1997). Interrupted axonal transport serves as a signal of potential cell damage, which consequently releases a number of molecules in the extracellular space to activate protective mechanisms. Released molecules are known as Damage Associated Molecular Patterns (DAMP) and include such important molecules as zinc, ATP and glutamate (Loane and Kumar 2016; Russo and McGavern 2016). These trigger calcium influx in the surrounding cells leading to oxidative stress, activation of glial cells and infiltration of leukocytes and neutrophils from the periphery. Traditionally, excessive release of glutamate has been known as excitotoxicity, however alternative studies suggest that there are also glutamate-independent mechanisms of excitotoxicity triggered after mTBI (Tehse and Taghibiglou 2018). Excitotoxicity is a transitional step from primary to secondary injury, which is triggered by prior pathological events and not by the impact itself. It

launches a biochemical cascade that can last for months and years post trauma and contribute to chronic impairments.

Neuroinflammation

A key element of secondary injury is inflammation, which is regulated by activated resident and peripheral immune cells infiltrated from the blood. Inflammation is a natural response to pathogens and stress, including mechanical impact, and serves to eliminate damage-associated molecules and restore normal balance of the environment. Resident immune cells include microglia and astroglia which are activated by excitotoxic molecules released during the primary injury. When activated, microglia undergo morphological changes from a resting ramified state to an amoeboid phenotype. Microglial activation is thought to have a dynamic temporal profile serving as a defense system in the acute phase and as neuroprotective at sub-acute time points (H. Xu et al. 2017). A simple categorization of activated microglia phenotypes describes two opposing states: pro-inflammatory (M1) and anti-inflammatory (M2) (Loane and Kumar 2016). M1 microglia are characterized by an upregulated expression of IL-1 β , TNF α , iNOS, IL-6 and IL-12p40 and are linked to oxidative stress, suppressed axonal growth, reduced phagocytic activity and chronic inflammation. M2 microglia express anti-inflammatory cytokines such as IL-10, IL-4 and contribute to neuronal regeneration, angiogenesis and remyelination. Typically, M1 is rapidly induced after TBI followed by the activation of M2 but under pathological conditions, the M1 phenotype remains dominant over M2 leading to long-lasting inflammation. For example, in mice subject to CCI, M2 microglia have been reported to peak at 5-7 days post injury while the increase in M1 microglia continued for 14-30 days post injury (Jin et al. 2012; Wang et al. 2013). Another study reported that transition from M2 to M1

begins at 7 days and can be found up to 1 year post-TBI (Loane et al. 2014). Prolonged activation of microglia results in pro-inflammatory cytokine production that suppresses regeneration and contribute to neuronal dysfunction (Loane and Kumar 2016; Xu et al. 2017). However, this dichotomic classification is considered an oversimplification and inappropriate to describe the complexity of microglial/macrophages responses after TBI (Loane and Kumar 2016; Kumar et al. 2016; H. Xu et al. 2017).

Like microglia, astroglial cells are also key components of the secondary injury. Healthy astrocytes support homeostasis of the brain by regulating the BBB (Abbott et al. 2006) and extracellular glutamate levels. Mechanical impact leads to astroglial proliferation, hypertrophy and their migration to the damaged tissue (Burda and Sofroniew 2014). Astrocytes move towards the injury site and elongate their processes forming a mesh-like structure called a glial scar (Bardehle et al. 2013). Glial scars are most evident in cases of focal injury, usually associated with moderate and severe TBI, however some studies showed the presence of such scars in diffuse mTBI (Burda et al. 2016). Glial scars segregate damaged and inflamed tissue from the adjacent cells and serve as a barrier that regulates spreading of the pathology (Wanner et al. 2013). The migration of astrocytes is triggered by matrix metalloproteinase 9 (MMP9) released by reactive astroglia into the extracellular space (Candelario-Jalil et al. 2009) and by aquaporin 4 (AQ4) translocating from the perivascular space to astrocytes (Ren et al. 2013). In the proximity to damaged tissue, astrocytes respond to and produce cytokines, chemokines, inflammatory mediators and DAMPs (Burda et al. 2016). Several mouse models of mTBI/r-mTBI showed an acute increase of GFAP starting 24h post-injury with the peak at days 3-7 followed by progressive increase during chronic period (Kane et al. 2012; Mouzon et al. 2014,2018; Susarla et al. 2014). Typically, immunohistochemical (IHC) analysis reveals GFAP signal in cortical and

subcortical areas in proximity to the injury site during the acute phase, while in more chronic periods GFAP is prevalent in the corpus callosum and hippocampus. Due to the vital role of these brain areas in memory formation, chronic inflammation may be associated with memory loss. Neuroinflammation has been demonstrated to be the most consistent pathology in response to experimental mTBI as was shown in all previously described animal models of mild injury.

Tau phosphorylation and oligomerization

There is a considerable interest in the investigation of the pathobiology of tau protein in pre-clinical models of mTBI, given its potential role in neurodegenerative diseases and aging. Tau is a microtubule associated protein that is responsible for microtubule stabilization and axonal transport. The tau protein is primarily located in the axonal cytoskeleton but can also localize to the nucleus, plasma membrane and post-synaptic density. Tau is expressed by *MAPT* gene that encodes a total of 16 exons, out of which exons 2, 3 and 10 undergo alternative splicing (for review see Wang & Mandelkow 2016). Exons 2 and 3 contain amino terminal sequences while exon 10 encodes carboxyl terminal repeats that function as microtubule binding domains. When exon 10 is present, there are 4 microtubule repeats (4R) and when absent, there are 3 microtubule repeats (3R) (McMillan et al. 2008). Human tau profile is represented by the total of 6 isoforms (0N3R, 1N3R, 2N3R, 0N4R, 1N4R, 2N4R) that vary by the presence or absence of N-terminal (0N, 1N or 2N) and C-terminal microtubule binding repeats (3R or 4R) (Sealey et al. 2017). In a healthy brain, the 4R:3R ratio is 1:1 while in neurodegenerative conditions, such as Alzheimer's Disease (AD) and CTE, the tau profile is predominantly 4R (Chen et al. 2010; Goedert and Jakes 1990; Schmidt et al. 2001). Iqbal and colleagues showed that in human brain, 4R tau had a higher level of phosphorylation than 3R, and 4R was demonstrated to acquire a

more favorable conformation for self-assembly and polymerization (Iqbal et al. 2009). Mild TBI, especially repetitive mTBI, has been shown to promote hyperphosphorylation of tau leading to its conformational changes and dissociation from the cytoskeleton (Iqbal et al. 2009; Morris et al. 2011; Fontaine et al. 2015). While tau phosphorylation is not always pathologic, in neurodegenerative disorders it is likely to form aggregates by binding normal tau (Alonso et al. 1996). These intracellular aggregates translocate to soma and dendrites and negatively affect synaptic functions and axonal transport. Phosphorylated tau can be released by exocytosis into the extracellular space (Morris et al. 2011) and spread in a prion-like behavior forming oligomers and tau threads that are hallmarks of AD and other forms of dementia, and are now linked to chronic effects of mTBI (Guo and Lee 2011; De Calignon et al. 2012).

Interestingly, tau pathology in wild-type rodents does not recapitulate the tau pattern in human brains. There are several reasons that can contribute to such differences. First, both human and mouse brains express 2 types of tau isoforms, 3R and 4R, however, the relative proportions of these isoforms vary between the species. A mouse tau profile is mostly represented by 4R forms, representing 98% of all tau, while humans express an equal ratio of 3R:4R (McMillan et al. 2008). Despite an increased 4R:3R ratio in wild-type mice, literature shows inconsistent results in tau behavior under pathological conditions varying from persistent to transient to no changes (for review see Ojo et al. 2013). One study compared single and repetitive mTBI (6 hits daily for 7 days) in C57BL/6 mice and observed an increase of tau phosphorylation (AT8) at 1 month but not at 6 months after single TBI (Petraglia et al. 2014). In the r-mTBI mice, however, p-tau was observed up to 6 months post injury. Meanwhile, in our previous study, wild-type mice, which received 5 mTBI 48 hour apart, did not exhibit phosphorylation of tau at any timepoint up to 24 months post TBI (Mouzon et al. 2018). Several

other studies confirmed acute/subacute accumulation of tau across different models of TBI including FPL (Hawkins et al. 2013) and blast injury (Goldstein et al. 2012), however publications extending these data to chronic timepoints post injury remain scarce. Second, human and murine tau differ in the number of amino-acid residues (where humans express 14 additional residues) that are crucial for protein conformation and its enzymatic activity (Jeganathan et al. 2008; Goedert and Jakes 1990; Andorfer et al. 2003). Taken together, the differences in the ratio of tau isoforms and amino acid residues can cause inconsistency between the mouse and human tau response to brain injury. Using transgenic mice that express humanized tau has been proposed (and adopted) as an approach to more closely mimic r-mTBI and possible CTE pathology in pre-clinical models.

A convenient model for tauopathies is the hTau mouse that expresses all 6 isoforms of human nonmutated tau on a null murine tau background (Andorfer et al. 2003). These mice are produced by crossing tau knock-out (KO) mice, with a targeted disruption of exon 1 of *MAPT*, with 8c mice expressing human tau transgene via a P1-derived artificial chromosome (PAC) cloning vector (Duff et al. 2000; Tucker et al. 2001). The first generation of mice expressing human tau is then backcrossed with KO mice to generate a homozygous line. hTau mice reproduce human-like tau pathology where tau translocates from axons to cell bodies by 3 months of age and becomes hyperphosphorylated by 6 months (Andorfer et al. 2003), and they develop age-dependent memory deficits and synaptic dysfunction by 12 months of age, while no other health problems linked to weight, basic reflexes, anxiety and motor functions are present (Polydoro et al. 2009). The most common areas of pathology in hTau mice are hippocampus and neocortex, including subcortical regions such as medial septum and the nucleus of the diagonal band. Moreover, these mice demonstrate thinning of corpus callosum, ventricular enlargement,

and reduced cell number between 10 and 14 months of age. This pathology is believed to be driven not only by the absence of mouse tau and the presence of human tau but also by a higher expression of 3R tau in hTau mice. Interestingly, injections of tau oligomers from mTBI (blast or FPI) hTau mice into healthy hTau mice cause cognitive deficits confirming a direct link between the pathologic tau and behavior dysfunction (Gerson et al. 2016). At the Roskamp Institute, we have used hTau mice to study the effects of repetitive mTBI on chronic pathological and cognitive outcomes that involve modifications of tau. First, we found that 18-month old exposed to r-mTBI but not to single mTBI have increased levels of tau phosphorylation. These data also demonstrated TBI-dependent tau pathology in aged (18-month old) hTau mice where tau pathology was already present at the time of injury (Ojo et al. 2013). Second, the apparent significance of TBI-dependent p-tau in humans, typically detected at times long past the period of TBI exposures, encouraged us to measure tau levels at 6 months post first TBI. For this, we administered r-mTBI for 3-4 months (2 hits per week) and at 3 months post-last injury observed a long-lasting increase in total tau and mild increase in phosphorylated tau (Ojo et al. 2016). Given that we have not demonstrated TBI-dependent tau pathology in WT mice on our laboratories, these findings confirm the benefits of using hTau mice over wild-type to replicate human-like TBI response.

1.5 Therapeutic approaches for TBI

1.5.1 Clinical Trials for TBI

Over the past several decades, more than 50 clinical trials have been initiated to test potential neuroprotective and neuroreparative pharmacological interventions for TBI (Stein et al. 2015). Unfortunately, all these trials failed during Phase II or Phase III and none of the tested

drugs have been approved for clinical application (Stein et al. 2015). Direct comparison of these trials is complicated due to a high variability of injury severities across patients, however, the main findings are worth mentioning.

The largest trial ever conducted on head trauma, to date, was CRASH (Corticosteroid Randomization After Significant Head injury) which was designed to measure the effect of the corticosteroid methylprednisolone on neurorecovery in patients with severe TBI (Roberts 2002; The CRASH trial protocol (corticosteroid randomisation after significant head injury) [ISRCTN74459797] 2001). Prior experimental studies in animal models confirmed the ability of corticosteroids to decrease intracranial pressure (ICP), inhibit immune response and reduce neurodegeneration (Edward D. Hall 1985; Ildan et al. 1995). Previously conducted clinical trials of corticosteroids in head injuries were too small to confirm or refute their clinical benefits (Alderson and Roberts 2011). Hence, the need for a large-scale study seemed rational. The hypothesis stated that a high-dose (0.4 g/h) infusion of corticosteroids for 48 hours could reduce mortality and disabilities in patients with severe TBI (Edwards et al. 2005). The trial aimed to recruit 20,000 subjects within an 8-hour post injury window and assess their outcomes at 6 months post treatment using Glasgow Outcome Scale (GOS). However, the trial was terminated when 10,000 individuals had been randomized to the trial, when the group allocated with corticosteroids was found to demonstrate increased mortality at 14 days and 6 months following the treatment (Edwards et al. 2005; Roberts et al. 2004). The increased death rates in the treatment groups did not differ by injury severity or time after injury. The main flaw of the study was determined to be a poor understanding of the proposed mechanism of action of the treatment, and the lack of a specific target. The CRASH study indicated that non-specific immune suppression acutely after TBI can be detrimental.

Another set of promising clinical trials investigated the effect of progesterone in TBI, due to numerous pre-clinical studies having shown its significant benefits (Siddiqui et al. 2016). The rationale for using progesterone was based on the observations of superior cognitive performance in naïve (uninjured) female mice and the ability to replicate the same behavior in male mice upon administration with the hormone (Auger and Forbes-Lorman 2008; Frye et al. 2009).

Progesterone was also shown to decrease inflammation, brain edema, apoptosis and axonal injury in animal models of TBI and ischemic injury (Guennoun et al. 2015; Chan et al. 2014; Chen et al. 2008; Lei et al. 2014; O'Connor et al. 2007). Two Phase II clinical trials demonstrated safety of progesterone injections in patients with mild to severe TBI (Wright et al. 2007; Xiao et al. 2008). Progesterone treated groups also showed decreased mortality and better functional outcomes for up to 6 months after head injury. These positive results prompted two independent Phase III clinical trials, ProTECT III (*Clinicaltrials.gov* NCT00822900) and SyNAPSe (*Clinicaltrials.gov* NCT01143064), were initiated to test acute administration of progesterone in patients with moderate and severe TBI (Stein 2015). In the ProTECT III study, progesterone was administered within 4 hours post-TBI for 96 hours (0.5 mg/kg/h tapered over the additional 24 hours, intravenously (i.v.)) in patients with GCS < 13, and the analysis included GOS, Disability Rating Scale (DRS), and mortality (Wright et al. 2014). In the SyNAPSe trial, patients with GCS < 8 received treatment within 8 hours post TBI for total of 120 hours (0.71 mg/kg/h for 1 hours following 0.05 mg/kg/h for 119 hours, i.v.) and the outcomes were assessed using GOS. Both studies analyzed TBI outcomes 6 months after the injury. In both studies, progesterone treated patients exhibited the same recovery rate as the placebo group and no differences in outcome compared to placebo group were detected (Skolnick et al. 2014; Butler

2015). Further attempts to test progesterone for TBI have been withdrawn due to the failures of these two independent trials.

Interestingly, some drugs that have been tested in TBI patients, but failed to show significant improvements, are still used as medications for brain injuries. For example, amantadine, a dopamine agonist, is one of the most common medications prescribed for TBI despite its failures in clinical trials (Hammond et al. 2015). In rats, amantadine increases dopamine release while reducing dopamine reuptake and has been shown to improve TBI-induced motor and cognitive deficits, including memory impairment and depression-like behavior (Tan et al. 2015; E. Y. K. Huang et al. 2014). Several clinical studies tested acute and sub-acute administration of amantadine in patients with severe TBI and showed accelerated pace of functional recovery during active treatment compared to placebo (Giacino et al. 2013; J. M. Meythaler et al. 2002). In the first study, patients that remained in the vegetative state (VG) or minimally conscious state (MCS) 4-16 weeks post TBI received amantadine (200-400 mg/day) for 4 weeks and the DRS score was measured at 2 weeks after the termination of treatment. Amantadine treated patients exhibited faster recovery during the 4 week period of active treatment whereas this effect disappeared during the following 2 weeks when treatment was not present (Giacino et al. 2013, *ClinicalTrial.gov* NCT00970944). In the second study, amantadine (200mg/day) was administered within 4-6 weeks post TBI to patients with GCS < 10 (J. M. Meythaler et al. 2002). The study included 2 groups each of which received either amantadine or placebo in the first 6 weeks and treatments were switched in the following 6 weeks. Results were analyzed at 6 and 12 weeks after the beginning of treatment and included GOS, Mini Mental State Examination (MMSE), and DRS. The study found a significant improvement in amantadine treated patients in both groups regardless of when the treatment was started.

However, when amantadine was withdrawn during the second 6 weeks and cognitive functions were assessed at 12 weeks, no improvement was recorded. Additional clinical studies were unable to replicate any significant benefits of amantadine on cognitive outcomes or functional recovery (Schneider et al. 1999; Hammond et al. 2018).

Another drug that had promising pre-clinical results and underwent several clinical trials was erythropoietin (EPO). EPO is essential for erythropoiesis and is used as an endogenous neuroprotective in the ischemic brain (Mallet et al. 2017). Low levels of EPO are expressed by neurons and astrocytes under normal conditions and are upregulated after CNS insult. Animal models of TBI confirmed the neuroprotective effects of EPO through suppression of glutamate toxicity, apoptosis, oxidative stress and a number of proinflammatory cytokines: IL-1 β , TNF α , ICAM1, CCL-2 (Xiong et al. 2008, 2010; Peng et al. 2014; Zhou et al. 2017). Several clinical trials tested acute EPO administration in patients with moderate to severe TBI and demonstrated no difference in outcomes (Liu et al. 2016). A clinical trial in Australia (ANZICS-CTG NCT00987454) that recruited 606 patients with moderate and severe TBI analyzed their outcomes at 6 months post injury and showed no treatment effect on mortality and neurological dysfunctions when treated within 24 hours post-TBI and for 3 weeks (Nichol et al. 2015). Moreover, EPO did not impact plasma levels of potential TBI biomarkers NSE, S100 β , UCHL-1, NF, nor did it improve cognitive outcomes (Nirula et al. 2010; Hellewell et al. 2018; Nichol et al. 2015).

A few clinical trials have “repurposed” approved drugs to target TBI outcomes such as depression and sleep deficits. For example, sertraline, a common mood-altering drug, has showed efficacy in preventing the onset of depressive disorder in patients across different TBI severities (Jorge et al. 2016) while armodafinil improved sleep deficits in patients with mTBI

and moTBI (Menn et al. 2014). While most clinical trials recruit patients with moderate and severe TBI, mTBI remains greatly understudied in the human population.

1.5.2 Preclinical treatment studies for mTBI

One approach to address the problem of these many failures in TBI clinical trials is to have more thorough investigation of potential therapeutics in translationally relevant preclinical models. In the past decades, experimental studies have investigated a large number of treatments strategies including target-specific and multimodal drugs; natural and synthetic compounds; noninvasive approaches, such as environmental enrichment and exercises, and engineered approaches, such as laser, sound and magnetic stimulations (Pearn et al. 2017). Most of these approaches have been tested in animal studies of moderate and severe TBI to model a TBI population with GCS < 12 who are typically enrolled in these clinical trials, with only a few to apply similar treatments for mTBI.

Inflammation

Regardless of the severity of injury, inflammation appears to be an integral part of TBI and hence is a popular target in preclinical treatment studies. Anti-inflammatory drugs may represent the largest group of therapeutics used in animal models. They include, but are not limited to, cytokine inhibitors, glucocorticoids, inhibitors of infiltration of the peripheral immune cells, and drugs with unknown anti-inflammatory mechanism such as minocycline, erythropoietin, resveratrol, statins and others (for review see Bergold 2016). Infiltrating monocytes and neutrophils are an integral part of the immune response to TBI, and their inhibition has been shown to suppress neuroinflammation in preclinical models. For instance,

blood-derived monocytes are recruited by C-C motif chemokine ligand 2 (CCL2) which binds its receptor CCR2 and promotes their migration to the injury site (Muessel et al. 2000). Deficiency in CCL2 or CCR2 has been associated with reduced inflammation and faster recovery after TBI, while acute administration of CCR2 antagonists in TBI models ameliorated long-term cognitive deficits (Semple et al. 2010; Morganti et al. 2015; Hsieh et al. 2014). Neutrophils express CXCR1/2 which, in a similar manner to CCR2 on monocytes, bind their respective ligand CXCL1/2 and migrate into the injured area of the brain. Mice deficient for CXCR1/2 show reduced neutrophil migration, decreased mortality and improved BBB permeability (Semple et al. 2010). Minocycline, a second-generation tetracycline, has also drawn lots of attention due to its proven safety in humans and neuroprotective properties in several neurodegenerative disorders (Plane et al. 2010). In CCI models, acute and subacute administration (30min-72 h) of minocycline improved motor deficits, decreased lesion volume, suppressed caspase-1 activity and microglial activation (Sanchez Mejia et al. 2001; Vonder Haar et al. 2013).

Fewer studies have been carried out in mTBI models, although recognition of the prevalence of concussive injuries in humans has begun to be reflected with more mTBI studies in the literature. As some aspects of mTBI share pathological features seen in more severe models, drugs that have been investigated in moderate to severe TBI are now being used in mild models. As mentioned above, minocycline is one of these drugs that has been administered to both: moderate to severe TBI and mTBI. For all TBI severity, it has shown efficacy in damping microglia activation and downregulating IL-1 β / MMP-9 if administered acutely after injury (Bye et al. 2007; Homsy et al. 2009; Siopi et al. 2012). A prolonged 2 week treatment of mTBI rats with minocycline confirmed downregulation of microgliosis and amelioration of neurological functions but did not induce neurogenesis in the injured brain (Ng et al. 2012). Interestingly, one

study suggests that minocycline treatment within 9 h post TBI has prolonged positive effect on memory improvement up to 3 months following drug administration while other studies only support its acute efficacy. Another strategy that has been employed is to selectively influence the cytokines profile by either promoting anti-inflammatory or inhibiting pro-inflammatory cytokines. For instance, administration of anti-inflammatory cytokine IL-10 increases neurological recovery and suppresses pro-inflammatory response in FPI animal model (Knobloch and Faden 1998) while inhibition of TNF α in the same model decreases inflammation and promotes neurological recovery (Rowe et al. 2018).

Neurogenesis

Multiple mTBI models have reported that excitotoxicity leads to apoptosis and a decrease in newly proliferating cells which are usually compensated by the activation of neurogenesis and active cell proliferation. The use of neural stem cells (NSCs) in treatment of TBI has gain enormous interest over the last decade. NSCs are attractive candidates for contributing to restoration of brain function through repair (neuroprotection) or reconstruction (cell replacement) after brain trauma. The working principle is that NSCs, through their intrinsic migration to sites of brain inflammation, will promote an environment that fosters cellular regeneration by mechanisms such as delivery of growth factors, suppressing inflammation, reducing axonal injury, and/or by differentiating into mature neural-lineage brain cells. Pre-clinical studies suggest that administration of NSCs and mesenchymal (MSCs) stem cells have a positive effect on neurogenesis in TBI models (Hasan et al. 2017). In rats exposed to TBI, NSCs administration decreased apoptosis, increased expression of synaptic proteins and ameliorated neurological functions acutely after the injury (Pang et al. 2017; Ma et al. 2011). Interestingly, a cell-free secretome from human MSCs also decreased apoptosis and promoted vascular endothelial

growth factor (VEGF) production when administered in TBI rats (Chuang et al. 2012, 2013). Due to less severe neuronal loss in models of mild injury, stem cells therapies are poorly described in mTBI animals. One study showed that NCSs administered after mTBI express higher levels of survival and proliferation than in mice with severe TBI (Shindo et al. 2006). These mTBI mice exhibit greater expression of neurotrophic factors and lower levels of GFAP signal than mTBI untreated or severe TBI treated groups. Moreover, transplantation of MSCs in mTBI rats for 14 days results in an increased number of newly formed neurons and astrocytes and improved neurological functions (Anbari et al. 2014).

Described therapeutics represent a small fraction of treatments tested in mTBI models, but it is worth mentioning that only few of them proceeded to the clinical trials and none, so far, showed successful clinical translation.

1.5.3 Problems of translation

Although an expanding number of animal studies suggest beneficial effects of multiple therapeutics in TBI models, clinical trials have failed to replicate these results. So far, there is no available drug to treat post-TBI outcomes. In addition to the complicated pathophysiology of the injured brain, significant obstacles arise from the translation of preclinical findings into clinical practice. Recent failures in TBI clinical trials drew lots of attention to the possible reasons behind these failures. They include choosing irrelevant pre-clinical models, poor experiment design, unjustified selection of dose/time window, low sample size and other factors.

A lack of depth and detail in preclinical studies has been cited as a leading reason for the failure in clinical translation (Diaz-Arrastia et al. 2013). There has been insufficient

investigation of injury heterogeneity, drug mechanism of action, target engagement, pharmacokinetics and pharmacodynamics, bioavailability, dosing and administration paradigms. One of the main criticisms is large differences in the neuroanatomical structure of small animals compared to humans. The most common animals used for TBI research are rodents, which are popular due to their availability, low cost, easy manipulation and short life-span. Rats and mice represent 90% of all experimental TBI research. Rodents express many human-like pathological patterns after TBI including acute transient primary injury and long-lasting heterogeneous secondary injury. Similar pathophysiology makes them an attractive model for studying molecular mechanisms of TBI and its correlation with cognitive performance. However, strong anatomical differences between rodent and human brains may lead to inaccurate replication of injury mechanisms. The gyrencephalic human brain contains numerous cortical folds - gyri and sulci. Evolutionarily, gyrification has been linked to an increased surface area to total mass ratio and an increased number of neurons (Sorby-Adams et al. 2018). It plays an important role in structural organization and functioning. Gyrencephalic brains also have a higher ratio of subcortical white to grey matter (Dubois et al. 2008). In cases of mTBI, the involvement of white matter in the injury-induced pathology is high. Particularly, the white matter is comprised of myelinated axons which are extremely vulnerable to mechanical impact and result in DAI. Moreover, mathematical models of brain heterogeneities (i.e. gyri and sulci) showed increased levels of the equivalent stress under loading conditions compared to homogeneous models (Cloots et al. 2008). In another words, gyri and sulci may lead to the stronger tissue deformation in gyrencephalic brain than in a lissencephalic brain. Meanwhile, the presence of the sulci focuses mechanical stress away from the cortical surface into the base of sulci while in a rodent brain stress propagates uniformly across the cortical superficial layers (Ho and Kleiven 2009).

Such redirection of stress is believed to be protective for the surface of cortex but increases abnormal pathology in the depths of the sulci. Indeed, in a CTE brain, accumulation of NFTs is often found in sulci, a pathology that we are unable to demonstrate in lissencephalic brain (McKee et al. 2009; Sorby-Adams et al. 2018).

Another explanation of the many failures in human clinical trials is that they were based on preclinical studies that had poor translational relevance. In other words, the many variables listed above (e.g. dose, administration, paradigm, PK/PD, bioavailability, target engagement, outcomes and of course the potential efficacy of the treatment in question) were insufficiently characterized in the animal model to enable successful translation to more complex and heterogeneous human patient populations (Schwamm 2014). For example, the CRASH trial (the first large-scale human TBI trial) was based on preclinical studies that did not resemble the final clinical trial design. In the clinical trial, TBI patients (GCS < 14) were treated for 48 hours starting within 8 hours post-TBI, and their outcomes were analyzed at 6 months (Ian Roberts 2002). Prior preclinical studies, however, had administered corticosteroid treatment immediately after the experimental injury (within 5 min) and analyzed neurorecovery within 1 hour (Ildan et al. 1995; Hall 1985). The two unsuccessful progesterone trials again provide a good example of how experiment design including dosing, treatment window, and prior optimization can influence the final outcomes. Both Phase III progesterone trials (ProTECT III and SyNAPse) were based on multiple pre-clinical studies that showed only modest improvement in cognitive functions in TBI animals treated with the hormone (Stein 2015; Schwamm 2014). The earlier stage Phase II clinical studies also did not show strong therapeutic effect in the human population. In fact, the ProTECT Phase II trial, which treated TBI patients in the first 3 days post injury and evaluated their cognitive symptoms at 30 days, found that both treated and non-treated

patients had poor neurological outcomes (Wright et al. 2007). Similarly, a Chinese Phase II trial showed only modest cognitive improvements in TBI patients treated with progesterone (Xiao et al. 2008). Phase II data weren't strong enough to bolster moving forward with the Phase III, but with no treatments available, the pressure to advance to Phase II prevailed over scientific method. Observations also demonstrated that 50% of placebo patients had favorable outcomes by the end of the trial, indicating the overall beneficial effect of medical care and attention in all tested subjects, which undermines the ability to separate progesterone-dependent effects (Xiao et al. 2008). Although one of the aims of a Phase II trial is to identify the optimal treatment dose, this was not sufficiently investigated in the progesterone Phase II studies (Stein 2015). The Phase II trials varied in several parameters including dosing (0.71→0.5 mg/kg/h vs 1→2mg/kg/day), administration routes (i.v. vs intramuscular (i.m.)) and vehicles (Intralipi vs camellia oil) (Schwamm 2014; Stein 2015). No consensus was formed on the optimal drug concentration to be used in the following trials. Consequently, instead of first optimizing the dosing, the Phase III trials chose the highest dose expecting to reach the maximum therapeutic effect. This strategy failed to demonstrate positive impact on cognition in TBI patients. The problem, however, began with inappropriate translation from the preclinical studies wherein a U-shaped dose response to progesterone was observed, with higher doses demonstrating lower efficacy (Goss et al.2003; Wali et al. 2014). In both studies, TBI rats underwent acute treatment with progesterone using three different doses - 8, 16 or 32 mg/kg. During the behavior assessment, only moderate doses, 8 and 16 mg/kg, were associated with cognitive improvement while the highest dose did not show similar therapeutic effect. These findings prove the lack of direct dose-effect correlation for individual drugs and must be accounted for when designing clinical trials. Another flaw of the progesterone trials was the inconsistency of the therapeutic

time window when compared to prior animal studies. In preclinical studies, the drug is usually given right after injury while in humans by necessity it will typically be delayed by at least a few hours (Diaz-Arrastia et al. 2013). This design fails to account for the interim period after the injury and before the patient seeks medical care. In fact, both progesterone trials administered the drug within the first 4-8 hours post TBI. This is an even greater translational problem in the case of mTBI as patients do not typically seek medical attention immediately after the injury due to the lack of gross symptoms.

According to medical records, only 10% of all TBI victims admitted to the ER in the US are diagnosed with mild forms of brain trauma (Laskowitz and Grant 2016). Taking into account that mTBI accounts for 70-90% of all brain injuries worldwide, such rates appear to be extremely low (Cassidy et al. 2004). Similarly, patients who get admitted to the ER are frequently misdiagnosed due to a general underestimation of mTBI by medical professionals. The GCS, which is the most popular assessment system, is a useful tool for differentiating moderate and severe forms of brain injury but is less helpful for distinguishing mTBI from non-concussed patients (Cappa et al. 2011). In fact, underestimation of mTBI, both by patients and medical attendees, creates a void in the literature by not including a large number of mTBI cases in the epidemiological and clinical studies (Laskowitz and Grant 2016). One population-based study performed in New Zealand was aimed to identify the annual incidence of TBI nationwide and showed higher rates of mTBI admissions in NZ (790 per 100,000 per year) than similar studies from other high income regions (Europe 47-453 per 100,000 per year; North America 51-818 per 100,000 per year) (Feigin et al. 2013). They reported that the advantage of their study was a thorough investigation of the overlooked diagnoses of less severe forms of injuries.

Due to the long-lasting consequences of TBI, the evolving pathobiology and competing mechanisms of neurodegeneration and neurorepair, and the heterogeneity of timing of first treatment, drugs that are effective in the acute phase may lose their therapeutic potential if administered at a delayed timepoint. A transition from primary to secondary injury results in the appearance and disappearance of potential drug targets. For example, glutamate release following primary injury leads to rapid activation of NMDA receptors and calcium overload whereas ongoing secondary injury produces downregulation of NMDA receptor expression (Guerriero et al. 2015; Mohamadpour et al. 2019). In the TBI models, NMDA antagonists were able to prevent calcium overload when administered within 24-72 hours post TBI (Shohami and Biegon 2014) while treatment with NMDA agonists at acute timepoints (8 and 16 hours post TBI) was not effective. Later administration of these agonists, however, improved synaptic plasticity and neurological functions (for review see Mohamadpour et al. 2019). A calcium channel blocker (CCB) ziconotide, administered in rats 15 min after TBI, improved mitochondrial functions while administration at 1 and 10h post TBI had no effect (Weber 2012). The anti-inflammatory drug minocycline also showed heterogeneous temporal effects in TBI models: it increased myelin content and improved spatial memory in CHI rats when administered at 12h and 24 h but not 72h after injury (Sangobowale et al. 2017, 2018). A recent review summarized 27 preclinical studies of different TBI models where treatment was administered no earlier than 12 hours after the injury (Mohamadpour et al. 2019). In 25 of them, the latest time of the initiation of treatment was 24 hours with only two extending their window up to 72 h or one month post-TBI. This intervention time may still not translate well to the many human cases where cognitive outcomes manifest starting several months or years after TBI. Attempts to relate timelines seen in humans to those used in animal models is crucial for successful translation.

1.6 Potential therapeutics developed by The Roskamp Institute

Identification of effective therapeutics for TBI remains a key challenge in the TBI research field. Despite existing attempts to suppress pathological mechanisms, none of the clinically tested treatments can effectively mitigate both metabolic and clinical deficits. In the Roskamp Institute, we have developed and characterized different mouse models of r-mTBI in collaboration with clinical consultants, in order to ensure that our models have translational relevance (described in Materials and Methods). To address the heterogeneity of human mTBI, we used different paradigms that varied in injury repetitiveness, age of mice and therapeutic time window. Through our work on AD and neuroinflammation we have characterized two drugs - nilvadipine and anatabine - both preclinically and clinically, which have relevance for TBI pathophysiology in terms of their effect on neuroinflammation, amyloid and tau. Although they appear to have different molecular targets, their multimodal actions on these pathobiologies have therefore suggested both drugs as candidate therapeutics for TBI.

1.7 Hypothesis

Given the current lack of any effective therapies for TBI, and the relative lack of investigation of any therapeutic approaches for *mild* TBI, my goal was to test the potential efficacy of two drugs previously tested at the RI: nilvadipine and anatabine. The obstacles to developing treatments for r-mTBI arise from the complexity of pathological mechanisms including their high heterogeneity and time-dependent changes. Previous studies have focused on targeting individual molecules involved in the pathogenesis of r-mTBI. We hypothesized that using drugs that influence several aspects of TBI pathobiology may reduce neurodegeneration and/or increase neurorepair. Therefore, in this thesis, I addressed the following aims: 1) To

replicate heterogeneity of human concussions by using two mouse models of r-mTBI; 2) To compare the effect of young vs old age in mice at the time of injury on the extent of TBI pathology and cognitive deficits; 3) To test two potential TBI therapeutics - nilvadipine and anatabine - that have demonstrated safety in humans; 4) To address the long-term consequences of mTBI by exploring delayed and chronic treatment paradigms; and 5) To investigate common therapeutic responses across these multiple approaches in order to identify viable, translational targets for further investigation.

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Chapter 2 – Investigation of the effect of acute treatment with nilvadipine in the aged hTau mice following r-mTBI

2.1 Introduction

2.1.1 Nilvadipine is a potential treatment for mTBI

The human neuropathology of r-mTBI has many facets, which are presumed to share common mechanisms with neurodegenerative diseases. For example, repetitive mild TBI (r-mTBI) and Alzheimer's Disease (AD) share similar pathological features such as the presence of neurofibrillary tangles (NFTs), a marked neuroinflammatory response, and to a lesser extent, the presence of amyloid- β (A β) pathology (Tateno et al. 2015). There are currently no FDA-approved drugs for the treatment of TBI, however the shared pathology may indicate that treatments tested for AD could be effective at treating the consequences of mTBI by reducing tau hyperphosphorylation and neuroinflammation. Unfortunately, there are no disease modifying treatments for AD and most strategies are targeted toward symptomatic therapy, but medical advancements in targeting AD pathology provide a wide range of potential therapeutic candidates (Weller and Budson 2018). Our scientists have previously shown that the anti-hypertensive drug nilvadipine can enhance A β clearance, decrease tau phosphorylation and reduce inflammation in a mouse model of AD (Paris et al. 2014). Nilvadipine is a dihydropyridine (DHP) class molecule that blocks L-type calcium channels and exhibits anti-hypertensive properties (Brogden and McTavish 1995). In preclinical studies, nilvadipine was first demonstrated to have neuroprotective properties in ischemic rats (Kawamura et al. 2005; Takakura et al. 1994). In addition to reduced apoptosis, it also improved spatial memory in stroke animal models (Iwasaki et al. 2003). When tested in AD models, nilvadipine decreased

A β production, increased A β clearance across the BBB and improved cognitive functions, whereas other DHPs such as nitrendipine and cilnidipine were less effective in reducing AD pathology, indicating that it is not a common class effect of the DHPs (Paris et al. 2011; Bachmeier et al. 2011). Additional studies proposed an alternative target for nilvadipine action – through inhibition of spleen tyrosine kinase (syk) (Paris et al. 2014). In the P301S transgenic mice, which develop filamentous tau lesions by 6 months and neurofibrillary tangles by 9-12 months, nilvadipine treatment resulted in the suppression of syk phosphorylation and reduced tau pathology (Paris et al. 2014). Further, a decrease of amyloid and tau pathology was also shown in P301S mice when they were treated with selective syk inhibitors (Paris et al. 2014). In two independent, small clinical trials, nilvadipine demonstrated therapeutic effect on cognitive function in hypertensive patients with mild cognitive impairment (MCI) (Hanyu et al. 2007, 2007(2)). In the first trial, 12 amnesic MCI patients with hypertension were randomly assigned to either amlodipine (6) or nilvadipine (6) for 12-16 week followed by cognitive evaluation and SPECT imaging (Hanyu et al. 2007). Nilvadipine, but not amlodipine, improved CBF in the frontal lobe and ameliorated working memory. Stronger therapeutic effects of nilvadipine were attributed to its ability to easily cross BBB compared to amlodipine and to its alternative anti-degenerative mechanisms. In the second study, a 20-month assessment conducted in 15 subjects with MCI (12 subjects from the previous study) treated with either nilvadipine or amlodipine showed that nilvadipine delayed clinical a progression of MCI to AD, with only 1 patient (out of 8) been converted to AD in the nilvadipine group while 5 (out of 7) were converted in the amlodipine group (Hanyu et al. 2007) .

Given the promising preclinical data from our laboratories, and independent supporting clinical data, we conducted an open label Phase I/IIa clinical study (in Europe, where the drug

was available on prescription for hypertension) to determine the feasibility of treating AD patients, and we showed safety and tolerability of nilvadipine in the AD patients and no adverse effects on blood pressure (Kennelly et al. 2011). We then investigated nilvadipine in a Phase III randomized controlled clinical trial for AD in Europe (NILVAD) that recruited 510 mild to moderate AD patients for treatment with nilvadipine or placebo (Lawlor et al. 2014). After 78 weeks of daily drug administration no differences in cognitive outcome measures were observed between treated and placebo groups for the entire cohort; however, when stratified according to baseline MMSE scores, the very mild AD cases (≥ 25 , N=80), demonstrated a significant reduction in cognitive decline following treatment with nilvadipine compared to placebo (Lawlor et al. 2018; Abdullah et al. 2019). These encouraging data, together with our preclinical data demonstrating the ability of nilvadipine to suppress inflammation, amyloid and tauopathy, pathologies common for both AD and TBI, encouraged us to test this drug in our models of r-mTBI. Moreover, proven safety and tolerability of nilvadipine makes it a promising candidate for successful translation to future TBI clinical trials.

2.1.2 Rationale for using aged mice of r-mTBI

We initially investigated the therapeutic efficacy of nilvadipine in our model of 5r-mTBI (5 hits, 48 h inter-concussion intervals) in aged mice that were 24-26 months old at the time of injury. The rationale for using very aged mice is the high incidence of mTBI in the elderly human population and the lack of preclinical work addressing this population. In the USA, TBI patients aged 65 and older have double the rate of hospitalizations compared to younger patients (<65yrs: 60.6 per 100,000; >65yrs: 155.9 per 100,000 (Coronado et al. 2005)), with ground falls and motor vehicle accidents (MVAs) as primary causes of brain trauma (Mak et al. 2012;

Thompson et al. 2006). Age alone is known to be associated with cognitive decline and dementia, but also significantly worsens mTBI-related outcomes (Bartres-Faz et al. 2001; LeBlanc et al. 2006; Lee et al. 2013; Mak et al. 2012; Onyszchuk et al. 2008; Thompson et al., 2006). Growing evidence links mTBI, and particularly r-mTBI, with neurodegenerative diseases such as AD (Lee et al. 2013; Tateno et al. 2015), PD (Jafari et al. 2013), ALS (McKee et al. 2010) and CTE (Gardner and Yaffe 2015; McKee et al. 2013). Persons aged 65 and older have a higher probability of developing these disabilities following mTBI due to pre-existing medical conditions, worsened functional recovery, and excessive pathological response (Mak et al. 2012). Additionally, the aged population is highly susceptible to repetitive brain injuries due to age-related deficits in motor functions, particularly balance (Lajoie and Gallagher 2004; Flanagan et al. 2006; Langlois et al. 2003). A single mTBI increases the probability of future falls, leading to repetitive concussions; and the cumulative injury effect in the elderly is thought to lead to more severe cognitive and pathological outcomes from similar injuries than in the young (Rothweiler, Temkin, and Dikmen 1998; Teo et al. 2018). Moreover, Simen and colleagues described a strong link between old age (>60 years old) and cognitive deficits including decline in episodic memory and executive functions (Simen et al., 2011).

At the time, we had an appropriate cohort of mice aged 24-26 months old which (given the typical laboratory lifespan of 30 months) is thought to represent old age in humans (>64 years old) (Dutta and Sengupta 2016). Following our 5r-mTBI or 5r-sham paradigm, we administered nilvadipine or vehicle for 21 days. Although our 5r-mTBI model does not demonstrate TBI-induced amyloid pathology in either wild type or hTau mice (Mouzon et al. 2014, 2018, 2018 (1)), neuroinflammation is a consistent feature of this model, and TBI-dependent effects on syk phosphorylation and tau have been reported within 15 days of injury in

both young and aged mice (Ferguson et al. 2017). These studies have also demonstrated that our models of r-mTBI induce deficits in spatial memory, motor functions, and disinhibition. Since such outcomes are representative of human r-mTBI and are often associated with an old age, we decided to measure cognitive functions in the current cohort. Moreover, understanding the correlation between the behavior and its underlying pathological changes is crucial to effectively assess the translational potential of nilvadipine.

2.1.3 Hypothesis:

- 1) Our model of 5r-mTBI will trigger neuroinflammation, tau pathology and cognitive deficits in the very aged (24-26 months old) hTau mice.
- 2) Nilvadipine [2mg/kg] administered for 21 days will suppress TBI-induced pathological and neurological outcomes in the aged mice.

2.2 Materials and Methods

2.2.1 Animals

Male and female hTau mice (Jackson Laboratories (Bar Harbor, ME)) were aged at the Roskamp Institute until 82-106 weeks old (weight 19-32g). The animals were housed under standard laboratory conditions (14-hour light/10-hour dark cycle, $23\pm 1^{\circ}\text{C}$, $50\pm 5\%$ humidity) with free access to food and water. All procedures were carried out under Institutional Animal Care and Use Committee (IACUC) approval and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2.2 Experimental groups and study design

A total of 39 mice were randomly assigned to 1 of 4 groups: sham-vehicle (n=9), sham-nilvadipine (n=9), r-mTBI-vehicle (n=10), r-mTBI-nilvadipine (n=11). Each group included both male and female mice. All animals underwent anesthesia for the same duration and frequency. Sham animals were allowed to recover in their home cages after each anesthesia, while r-mTBI mice received 5 injuries over 9 days with a 48-hour inter-concussion interval, using our well-established injury paradigm (Mouzon et al. 2012, 2014, 2017, Ojo et al. 2015; Tzekov et al. 2014, 2016). Either nilvadipine or vehicle (PBS:PEG 1:1) were injected intraperitoneally (i.p.) daily for 21 days, with the first injection administered immediately following the last anesthesia (in 5r-sham) or the last injury (in 5r-mTBI). Behavior tests started on the second day of injections and included Rotarod, Barnes Maze, and Elevated Plus Maze, as shown in Figure 1. Euthanasia was performed on day 22 after the last injection. Brains were further analyzed by immunohistochemical and biochemical methods for inflammatory markers, tau, and

phosphorylated syk. Researchers were blind to animal group assignments during both neurobehavioral experiments and immunohistochemistry.

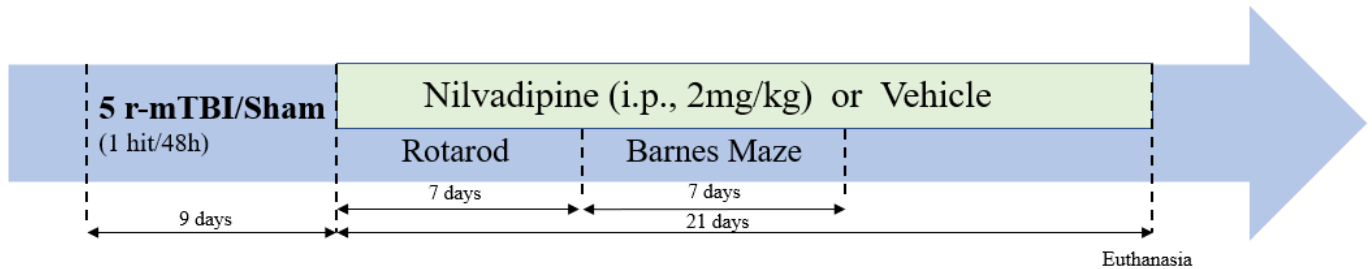


Figure 1. Study design

2.2.3 Injury Protocol

R-mTBI injury was performed as previously described (Mouzon et al. 2012, 2014; Tzekov et al. 2014, 2016; Ojo et al. 2015). All animals were anesthetized with 1.5 L/min of oxygen and 3% isoflurane prior to r-mTBI or sham injury. The heads were shaved and mice were placed on a heating pad to maintain body temperature at 37°C to prevent hypothermia. The head of each animal was fixed in a stereotaxic frame, and the blunt impactor tip (3 mm diameter) was positioned midway to the sagittal suture. The injury was triggered at 5 m/s velocity and 1.0 mm depth, with a dwell time of 200 milliseconds, using a myNeuroLab controller device (Impact One™ Stereotaxic Impactor, Richmond, IL). All mice experienced short-term apnea (<20 sec) and showed no skull fractures. All animals were allowed to recover from anesthesia on a heating pad and then returned to their cages with water and soft food access. Sham animals received anesthesia alone for the same duration of time as the r-mTBI mice, to control for the effects of repeated anesthesia. Mice were monitored daily for any abnormalities in behavior.

2.2.4 Treatment

All mice received either nilvadipine or vehicle via i.p. injections for 21 days, starting immediately after the last injury (the first injection was administered while animals were still under anesthesia). The site for i.p. injection was sterilized with alcohol and was changed every day to avoid discomfort due to repeated injections at the same site. The treated groups received 2mg/kg of nilvadipine dissolved in a 1:1 solution of PBS and PEG (polyethylene glycol) vehicle solution (Paris, Ait-Ghezala, et al. 2014). This dose is equivalent to the human dose of 8 mg that was prescribed in Europe conferring an antihypertensive activity. The animal equivalent dose (AED) was calculated using a conversion protocol based on the exponent of the body surface area (K_m) with account for differences in metabolic rate: $AED (mg/kg) = Human\ dose (mg/kg) \times K_m\ ratio$. $8\ mg/60kg \times 12.3 \approx 2\ mg/kg$ (Nair and Jacob 2016). The injection volume (100 μ l) was calculated based on the average animal's weight (0.028kg). Untreated animals underwent the same procedure, but received vehicle solution only (PBS:PEG/1:1). The nilvadipine and vehicle solutions were freshly prepared every day before the injections.

2.2.5 Motor function assessment

Motor function was assessed using the Rotarod apparatus, and the latency to fall from an accelerating rotating rod was measured. Baseline performance was recorded 1 day prior to the first injury/sham. Rotarod assessment started on the day after the last injury/sham procedure, and was carried out on days 1, 3, 5 and 7 post-last r-mTBI. An acclimation period involved 3 trials with a duration of 3 min each, and a 3 min rest interval in the animal's home cage between the trials (velocity = 5rpm, no acceleration). The mice were placed back on the bar during the acclimation period if they fell. All experimental trials, including the baseline trials, lasted for 5 min and were conducted with acceleration from 5 to 50 rpm over the 5 min period. Each animal

underwent 3 trials per day, with a 3 min rest interval between each trial. The fall time of each mouse was recorded in seconds. To ensure that fall time would correlate with motor coordination, rather than purely grip strength, if a mouse clung to the bar for more than 5 consecutive rotations on the accelerating rod without walking or making forward progress against the rotation of the bar, the time of the 5th rotation was considered to be the fall time and was recorded as such.

2.2.6 Cognitive function assessment

Barnes Maze (BM) was used to measure learning and short-term memory. BM was initiated on day 8 post-last mTBI/sham and lasted for 7 consecutive days to assess cognitive function. For 6 days, animals were trained to find the target hole which had a black escape box underneath. The walls in the room were equipped with visual cues and the brightness of the room was consistent throughout testing (7 days). The BM table was 1.2 meters in diameter and has 18 equally spaced holes around the perimeter. Every mouse had 4 acquisition trials per day, with a duration of 1.5 minutes each. The starting position for each trial during acquisition rotated, beginning at one of 4 cardinal directions of the maze and rotating, first clockwise (until reaching the initial position again) and then counterclockwise. If an animal did not find the target hole or did not go inside the box within the time limit, the mouse was guided to the target hole by hand. Regardless of their success, mice then spent 30 seconds in the box before returning to their cage. On the last day, 24 hours following the final acquisition trial, a probe trial was conducted during which the animals were placed in the middle of the maze and had 60 seconds to find the target hole, from which the escape box had been removed. The cumulative distance from the target hole, total distance travelled, time to find the target hole, and velocity were calculated using Noldus Ethovision XT software and analyzed to assess spatial memory and learning. Cumulative

distance is measured as the sum of the distance between the center point of the mouse and the center of the target hole over every sample from each trial at 30 samples per second. This distance stops accumulating when the trial ends either when time has elapsed or when the mouse enters the target box. Data are presented as the raw values of the cumulative distance.

2.2.7 Euthanasia

Animals were euthanized on the day after the last injection (22 days post-last injury/sham). Mice were anesthetized with 3% isoflurane and perfused transcardially with phosphate-buffered saline (PBS), pH-7.4. After perfusion, the brains were post-fixed in a solution of 4% paraformaldehyde (PFA) at 4°C for 48h and paraffin-embedded for immunohistochemistry.

2.2.8 Tissue processing and immunohistochemistry

Brain samples fixed in PFA were processed in paraffin using the Tissue-Tek VIP (Sakura, USA). Sagittal sections were cut at 8 µm using a Leica RM2235 microtome and mounted on positively charged glass slides. Prior to staining, sections were deparaffinized in xylene and rehydrated in ethanol solutions of decreasing concentrations.

Non-fluorescent staining for GFAP: Following rehydration, slides were processed with hydrogen peroxide for 15 min and heated in the citric acid buffer (pH-6) for antigen retrieval. Slides were then blocked with normal goat serum, washed with PBS, and incubated in a primary antibody for GFAP overnight at 4°C (GFAP7857983, Aves Labs, Inc., 1:10,000). On the next day, slides were processed using the anti-chicken VectaSTAIN ABC Kit and developed with 3,3'-Diaminobenzidine (DAB) before mounting.

Non-fluorescent staining for Iba1: Reactive microglia were stained using an anti-Iba1 antibody (ab107159, Abcam). After rehydration, slides were processed with hydrogen peroxide for 15 min followed by antigen retrieval using citric acid buffer (pH-6). Next, slides were blocked with rabbit serum for 1 h at room temperature and then incubated with the primary antibody (1:1000) overnight. On the next day, samples were processed using the anti-goat VectaSTAIN ABC Kit and developed with DAB.

Fluorescent staining for Iba1/p-tau/psyk: Fluorescent staining was performed with the antibodies for microglial marker Iba1 (ab107159, Abcam), phosphorylated tau at Thr231 (RZ3, Dr. Peter Davies) and at Ser202 (CP13, Dr. Peter Davies), and phosphorylated spleen tyrosine kinase psyk (Tyr525/526) (2710S, Cell Signaling). Following rehydration, antigen retrieval was performed by heating slides in citric acid buffer for 7 min in a microwave oven. Next, slides were washed with phosphate-buffered saline (PBS) and transferred to a Sudan Black solution for 15 minutes to prevent autofluorescence. Slides were then blocked for 1 h with 10% donkey serum solution in PBS, and primary antibodies for Iba1 (1:300), RZ3 (1:400), CP13 (1:400) and psyk (1:200) were applied overnight. On the next day, secondary antibodies AlexaFluor488 (A21202, Life Technologies), AlexaFluor568 (ab175477, Abcam), AlexaFluor 488 and AlexaFluor647 (A21449, Life Technologies) were applied for psyk, Iba1, RZ3 and CP13 respectively. Slides were mounted with ProLong Gold Antifade 4',6-diamidino-2-phenylindole (DAPI) Mount. Each marker was stained separately except for the double staining for Iba1/psyk that was performed to evaluate colocalization of the two.

2.2.9 Imaging

Imaging of *non-fluorescent* samples stained for GFAP/Iba1 was performed on an Olympus DP72 microscope at 10x magnification. Further analysis of the images included quantification of GFAP and Iba1 signal using ImageJ. Images were separated into individual color channels (hematoxylin counterstain and DAB chromogen) using the color deconvolution algorithm (Ruifrok and Johnston 2001). Three nonoverlapping regions of interest (ROI) of 100 μm^2 per image were then selected for the hippocampus, the body of the corpus callosum and the cortex (under the injury site). ROI were selected in the similar regions of the analyzed brain areas between the samples regardless of the obvious presence or absence of the pathological signal. A coverage area (%) per ROI was calculated and the mean value for each animal was used for further statistical analysis.

Fluorescent imaging was carried out using a confocal microscope (LSM 800 Zeiss) at 63x magnification. Cerebellum, hippocampus and cortex were screened to find areas of interest such as activated microglia, activated astroglia, p-tau and accumulation of psyk. Z-stacks were recorded for every image and orthogonal projections were obtained to enable a 3D representation of the picture.

2.2.10 Statistical analysis

All experimental data were analyzed using JMP 12 and GraphPad Prism 6 software. The data were checked for normality using Skewness-Kurtosis and Goodness of Fit. If normal, parametric method one-way ANOVA was applied to calculate the significance in the tested groups (p-values less than 0.05 were considered significant). If significant, post-hoc analysis was applied using Turkey's multiple comparison test/ Honest Significant Difference (HSD). The Turkey's test compares all possible pairs of means between different treatment groups and is considered significant if $p < 0.05$. The Shapiro-Wilk test was used if data were not normally

distributed. All data were transformed to logarithm or square root, when required, to reach normality before further analysis. Repeated-measure analysis of variance (MANOVA) was used to analyze continuous performance of mice in the Barnes Maze and Rotarod ($p < 0.05$ is significant). Error bars represent the standard error of the mean.

2.3 Results

2.3.1 Behavior assessment

All animals showed a decrease of the cumulative distance to the target hole over the course of the acquisition trials (Fig. 2A). The sham-vehicle group showed a 36% decrease from day 1 to day 6 while the r-mTBI-vehicle mice had a 28.5% decrease. R-mTBI-vehicle mice performed worse compared to the sham-vehicle over a 6-day period ($p < 0.05$, MANOVA). Treatment with nilvadipine in r-mTBI mice induced a 41% decrease of the cumulative distance from day 1 to day 6. Nilvadipine treated r-mTBI mice had a significant decrease in cumulative distance compared to the vehicle-treated r-mTBI mice ($p < 0.001$, MANOVA). The performance of sham-vehicle and sham-nilvadipine mice were not different from each other ($p > 0.5$, MANOVA). Distance traveled was decreased in all groups for at least 21% between days 1-6, however there was no differences in the extent of this decrease between the groups (Fig. 2 B).

The mean latency to enter the target box was only significantly decreased (18.6%) in r-mTBI-nilvadipine mice from day 1 to day 6 but not in the other groups (Fig. 2 C). This decrease was significantly different from the r-mTBI-vehicle mice (day 6 of acquisition: r-mTBI-nilvadipine 69.7 sec vs r-mTBI-vehicle 87.3, $p < 0.001$, one-way ANOVA). Surprisingly, we did not see any difference in latency to enter target box between groups on day 4. Behavioral testing data is inherently noisy and typically requires large n numbers to achieve statistical significance, thus the lack of an effect on latency during an individual day should not be interpreted as a lack of an overall effect during testing. Unexpectedly, probe data showed no difference in spatial memory between sham-vehicle, sham-nilvadipine and 5r-mTBI-vehicle groups ($p > 0.05$, one-way ANOVA) (Fig. 2D). However, 5r-mTBI-nilvadipine mice showed significant improvements in

finding the target hole compared to r-mTBI-vehicle animals ($p < 0.05$, one-way ANOVA). Injured mice treated with nilvadipine required less time overall (mean = 13.75 ± 6.9 s) to locate the target hole within the trial compared to other groups: r-mTBI-vehicle = 38.27 ± 4.4 s, sham-vehicle = 26.87 ± 5 s, sham-nilvadipine = 20.51 ± 8.4 s. There was no significant effect of nilvadipine treatment on motor function or anxiety level as measured by Rotarod and EPM (Fig.2E, F).

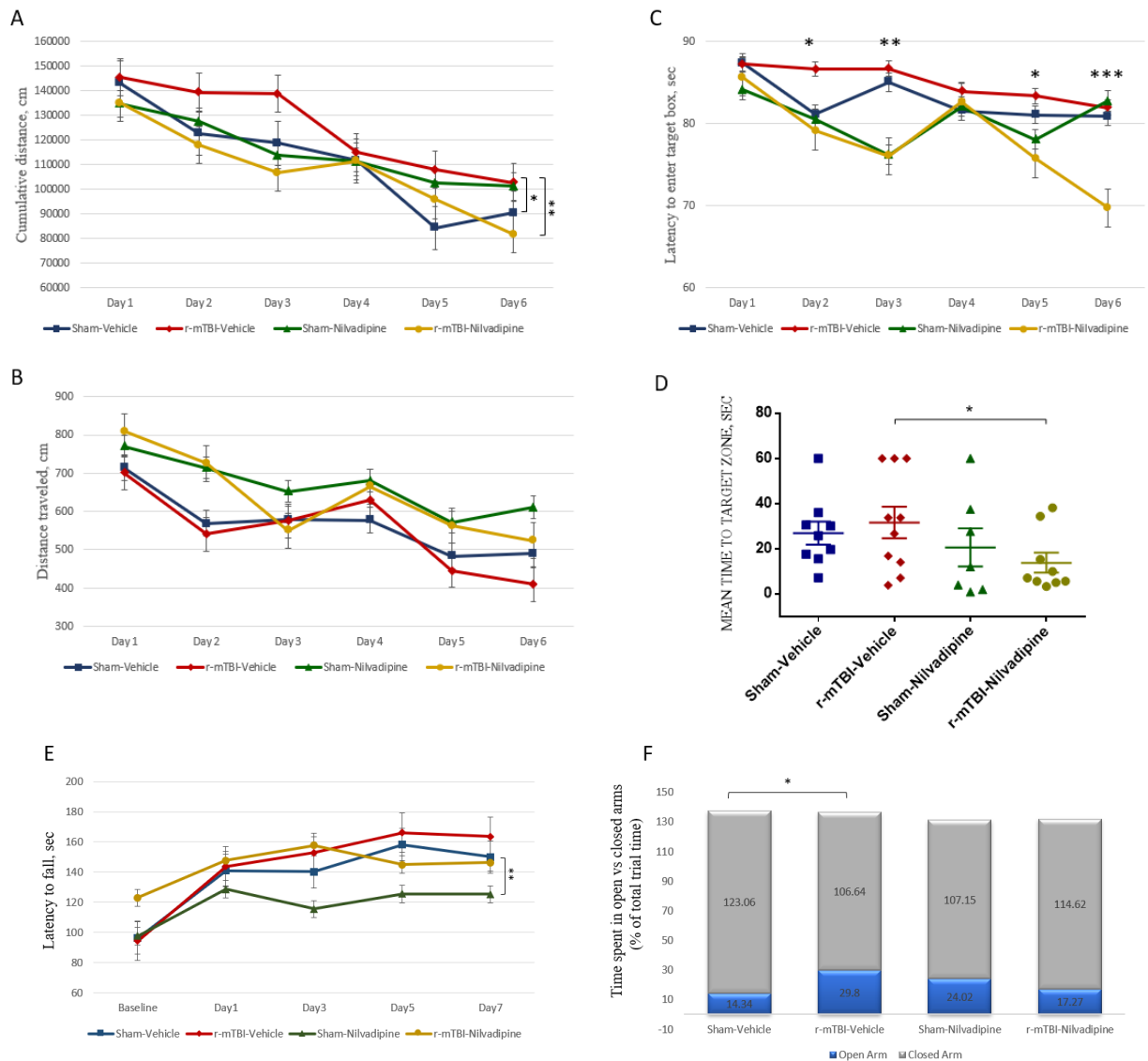


Figure 2. Behavior evaluation. Assessment of learning and spatial memory at 7 days post-last mTBI using Barnes Maze (A-D). Acquisition data shows a learning trend in mice by the mean cumulative distance to the target hole in cm (A) and the mean time spent to enter the target hole in seconds (C). There was no difference between the groups in the distance traveled (B). R-mTBI-Nilvadipine mice performed better than r-mTBI-vehicle group (cumulative distance $p < 0.01$, MANOVA; mean time: day 2 $p < 0.05$, day 3 $p < 0.01$, day 5 $p < 0.05$, day 6 $p < 0.001$, one-way ANOVA). Probe data shows spatial memory by the mean time to locate the target hole (sec) after 6-day training (D). r-mTBI-Nilvadipine group also performed better than r-mTBI-vehicle

mice ($p < 0.05$, one-way ANOVA). No significant differences were observed between r-mTBI-vehicle and r-mTBI-Nilvadipine groups for motor functions (E) and disinhibition behavior (F).

2.3.2 Immunohistochemistry

For mice subjected to r-mTBI, immunostaining for GFAP revealed evidence of a severe reactive astrogliosis in the corpus callosum (percent area: r-mTBI-vehicle $9.03\% \pm 1.05$ vs sham-vehicle $5.01\% \pm 1.2$, $p < 0.05$, one-way ANOVA with post-hoc Turkey's test) and in the region of the cortex surrounding the injury site (percent area: r-mTBI-vehicle $4.02\% \pm 2.09$ vs sham-vehicle $0.08\% \pm 0.09$, $p < 0.001$, one-way ANOVA with post-hoc Turkey's test) (Fig.3). After 21 days, significantly decreased GFAP immunoreactivity was observed in the corpus callosum (r-mTBI-nilvadipine $6.3\% \pm 1.3$ vs r-mTBI-vehicle $9.03\% \pm 3.2$; $p < 0.01$, one-way ANOVA with post-hoc Turkey's test) of nilvadipine r-mTBI versus vehicle r-mTBI mice. Treatment with nilvadipine also decreased GFAP immunoreactivity in cortex of r-mTBI mice (r-mTBI-nilvadipine $1.75\% \pm 0.31$ vs r-mTBI-vehicle $4.02\% \pm 2.09$; $p < 0.05$, one-way ANOVA with post-hoc Turkey's test).

A similar response was detected with anti-Iba1 staining, which indicated excessive TBI-dependent microgliosis in the corpus callosum (percent area: r-mTBI-vehicle $7.02\% \pm 2.2$ vs sham-vehicle $3.06\% \pm 0.7$, $p < 0.0001$, one-way ANOVA with post-hoc Turkey's test) and successful mitigation of this astrogliosis after treatment with nilvadipine (percent area: r-mTBI-vehicle $7.02\% \pm 2.2$ vs r-mTBI-nilvadipine $3.1\% \pm 2.5$, $p < 0.05$, one-way ANOVA with post-hoc Turkey's test) (Fig. 4). No difference between the groups was observed for Iba1 in cortex (Fig. 4 C).

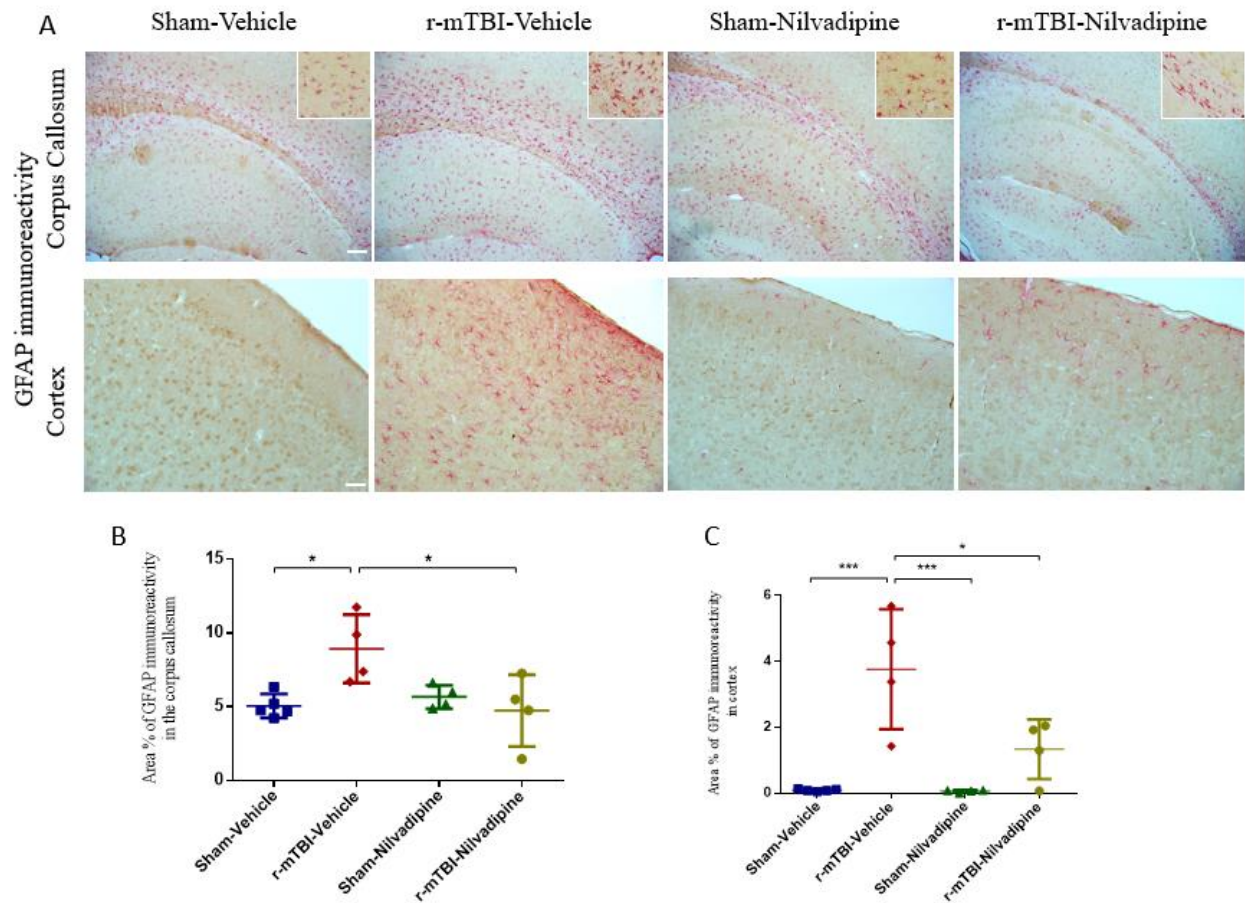


Figure 3. Evaluation of the effect of nilvadipine after r-mTBI on astrocytes (GFAP) at 22 days post-r-mTBI in the corpus callosum (CC) and cortex. A – representative images (10x) of the GFAP staining in the CC in different treatment groups show excessive gliosis after r-mTBI which was reduced by nilvadipine. In cortex, r-mTBI caused an increase in GFAP immunoreactivity surrounding the injury site ($p < 0.001$) which was attenuated by nilvadipine ($p < 0.05$). Quantitative analysis of GFAP staining in three $100\mu\text{m}^2$ fields of the CC (B) and cortex (C) at 22 days post-injury confirms injury-induced glial activation in the CC ($p < 0.05$) and cortex ($p < 0.001$) and a nilvadipine-dependent decrease of GFAP. Scale bars equal $100\mu\text{m}$ for CC and $50\mu\text{m}$ for cortex images. Data are presented as mean \pm standard error of the mean; significance is calculated using one-way ANOVA with post-hoc Turkey's test.

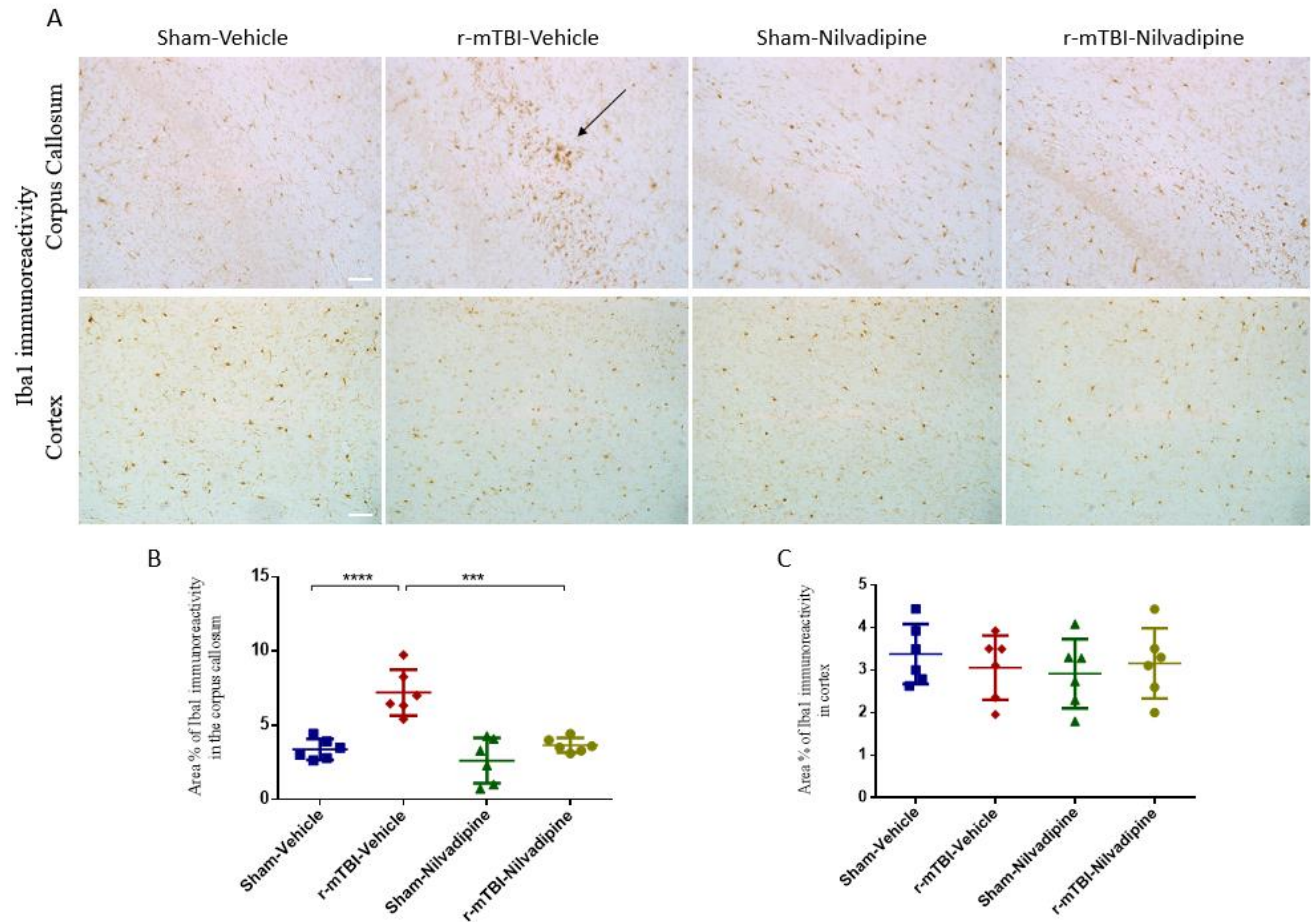


Figure 4. Evaluation of the effect of nilvadipine after r-mTBI on microglial population (Iba1) at 22 days post-r-mTBI in CC and cortex: A - representative images (20x) of the Iba1 staining in the CC revealed increased microglial activation after r-mTBI and a reduction of Iba1 immunoreactivity by nilvadipine. No difference in Iba1 was detected for cortex ($p > 0.05$) (A, C). Quantitative analysis of Iba1 staining in three $100\mu\text{m}^2$ fields of the CC (B) at 22 days post-injury confirms the injury-induced ($p < 0.0001$) and nilvadipine-induced ($p < 0.001$) changes in glial response in CC. Scale bars equal $50\mu\text{m}$. Data are presented as mean \pm standard error of the mean; significance is calculated using one-way ANOVA with post-hoc Turkey's test.

Tau phosphorylation at Thr231 and Ser202 is prominent in hTau mice and leads to an impaired tau-microtubules binding, which was shown to be increased in our r-mTBI model

(Mouzon et al. 2012, 2014, Ojo et al. 2013). Hence, the antibodies for these epitopes, RZ3 (Thr231) and CP13 (Ser202), were chosen for IHC in the current study. Fluorescent IHC for phospho-tau Thr231 (RZ3) revealed no significant injury effect in the CA1 area of hippocampus ($p>0.05$, one-way ANOVA with post-hoc Turkey's test) but there was an increase of RZ3 signal in cortex ($p<0.05$, one-way ANOVA with post-hoc Turkey's test) (Fig. 5). Despite the lack of the injury effect in CA1, quantitative analysis of the signal intensity confirms the reduction of RZ3 by 50% in r-mTBI-nilvadipine ($1,564\% \pm 107.6$) vs r-mTBI-vehicle mice ($3,715\% \pm 430$) (Fig.5 B; $p<0.05$, one-way ANOVA with post-hoc Turkey's test). Treatment with nilvadipine also decreased immunoreactivity for RZ3 in cortex (Fig. 5 C; $p<0.05$, one-way ANOVA with post-hoc Turkey's test). However, no difference between the groups was detected for phospho-tau Ser202 (CP13, by Dr. Peter Davis) in hippocampus or cortex (Fig.5D, E).

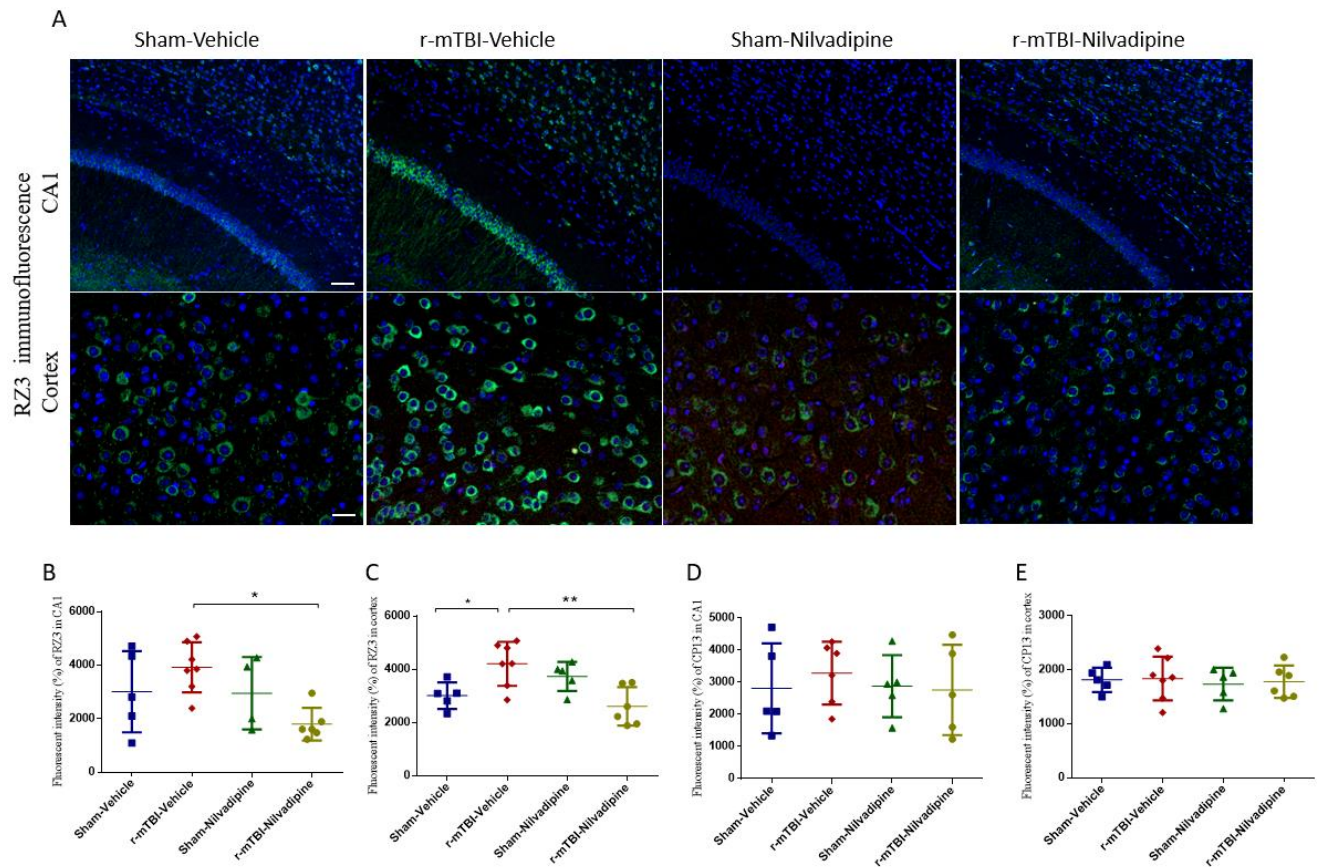


Figure 5. Evaluation of the effect of nilvadipine on **RZ3** immunofluorescence in CA1 and cortex. A - IHC shows a trend of increased **RZ3** immunoreactivity after r-mTBI in both CA1 and cortex. Quantitative analysis of the % intensity in the CA1 shows that **RZ3** intensity is slightly increased in r-mTBI-vehicle vs sham-vehicle mice but not significant ($p>0.05$). C - In cortex, **RZ3** is increased in the r-mTBI-vehicle mice compared to the sham-vehicle. Nilvadipine reduces **RZ3** fluorescent intensity in the CA1 by 50% ($p<0.001$) and in cortex by 30% ($p<0.05$). Scale bars equal 50 μm . No difference was shown between the groups for CP13 in CA1 (D) and cortex (E). Data are presented as mean \pm standard error of the mean; significance is calculated using one-way ANOVA with post-hoc Turkey's test.

Injured mice also demonstrated the presence of activated (phosphorylated) syk compared to the sham-vehicle group. Qualitative analysis revealed accumulation of psyk in certain sections of corpus callosum, but not uniformly across the brain. However, in these sections, psyk was colocalized with reactive microglia (Iba1), indicating a strong link with neuroinflammation (Fig. 6). Treatment with nilvadipine ameliorated psyk and Iba1 in the r-mTBI mice.

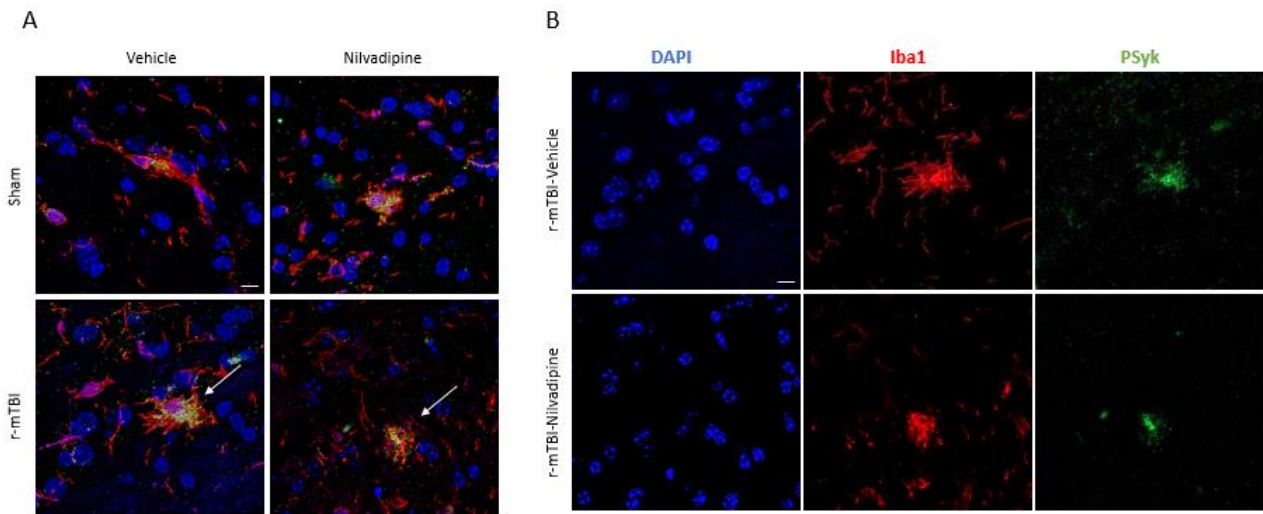


Figure 6. Evaluation of the effect of nilvadipine on **psyk**. A -Immunofluorescent staining of the corpus callosum with **DAPI**, **Iba1** and **psyk** revealed an increased activity of psyk in the r-mTBI-vehicle brains (white arrow). Upon treatment with nilvadipine, psyk signal was decreased to sham

levels (white arrow). B - Fluorescent immunohistochemistry for **psyk**/**Iba1**/**DAPI** revealed a colocalization of psyk and Iba1, indicating a strong link between activated syk and neuroinflammation. Mitigation of the psyk signal was correlated with decreased Iba1. Scale bars equal 10 μ m.

2.4 DISCUSSION

The effect of traumatic brain injury on aged animals and the ability of the aged brain to respond to the injury is remarkably understudied. Age is known to slow neurorehabilitation and prolong inflammation, which can worsen the pathogenesis of mTBI, exacerbate cognitive impairments, and increase the probability of developing dementia (Bartrés-Faz et al. 2001; Bernhardt et al. 2015; Hebert et al. 2003; Primiani et al. 2014). Our previous studies showed that the anti-hypertensive dihydropyridine, nilvadipine, is a syk inhibitor which can decrease neuroinflammation and amyloid pathology in the PSAPP transgenic mouse model of AD, and tau phosphorylation in the P301S mouse model of tauopathy (Paris et al. 2014). Here, we tested the effects of nilvadipine in hTau mice with r-mTBI to assess its effect on pathological and cognitive outcomes post-injury.

With regard to the neuropathological findings, consistent with our previous work using this 5r-mTBI paradigm, we observed increased GFAP and Iba1 immunoreactivity in the corpus callosum and in the area of the cortex beneath the impact site, even in these significantly aged mice. Our previous studies, wherein we have injured mice ranging from 3 to 18 months old, have demonstrated robust 5r-mTBI-dependent neuroinflammation regardless of the age of the mice at the time of injury (Ojo et al. 2013; Lynch et al. 2016; Mouzon et al. 2014, 2018; Ferguson et al. 2017). Both, young and aged groups of injured mice are characterized by reactive

astrogliosis and microgliosis in the corpus callosum, hippocampus and cortex. Our data confirm the persistence of TBI consequences over the lifespan of mice injured young, and despite the inherent age-dependent increases in neuroinflammation an injury effect is evident even in very aged mice. However, we did not observe any significant increase in tau phosphorylation following r-mTBI. Previous findings showing that tau phosphorylation increases with age in hTau sham animals (Andorfer et al. 2003; Polydoro et al. 2009; Ojo et al. 2013) are corroborated by our recent study which showed an overall increase of phosphorylated tau in aged (12 months old) when compared to our previously-studied young (3 months old) hTau mice (Ferguson et al. 2017). Thus, we hypothesize that the age-dependent increases in tau pathology may be obscuring any additional increases due to r-mTBI.

Regarding behavioral data, we did not detect an injury effect on motor function or anxiety-like behavior, and only a minor effect on spatial memory, which is likely due to the much older age of the animals at the time of injury in this study compared to our previous work. Our previous studies with the same injury paradigm in younger animals, aged between 3 and 12 months old at the time of injury, resulted in memory impairment after 5r-mTBI (Mouzon et al. 2012, 2014; Ferguson et al. 2017). However, our previous data also show that in mice injured at a young age (2-3 months) cognitive deficits are less apparent over time, particularly when a year or more has elapsed post-injury (Mouzon et al. 2014, 2018; Ferguson et al. 2017). Thus, aging plays a confounding role in the baseline behavioral levels, so it obscures the effects of injury. Few studies have investigated the effects of r-mTBI on aged mice, and this study is the first to utilize this 5r-mTBI paradigm in such old mice. We propose that the advanced age of these animals causes detrimental deficits resulting in a “ceiling effect” that prevents a clear differentiation between age and injury-dependent deficits. In support of this hypothesis, studies

of hTau vs WT mice have shown that the hTau mice develop age-dependent memory deficits, not present at 4 months, but evident by 12 months of age (Polydoro et al. 2009; Yin et al. 2016). The characteristics of hTau mice aged 24 months have not previously been reported, but we would reasonably conclude that such mice would have dramatically impaired cognitive performance. Indeed, even in comparing sham mice across our studies we observed decreased performance in cognitive tasks with age; e.g. in our study of hTau mice aged 3 or 12 months, cognitive performance was diminished in the older versus young sham animals (Ferguson et al. 2017); while in cohorts of wild type mice aged to 27 months, we saw a progressive worsening of memory throughout life (Mouzon et al. 2018). Thus, the age-dependent pathology underlying spatial memory (not specifically investigated here) appears sufficient to mask any additional effects of injury.

Simen and colleagues reviewed a plethora of clinical and preclinical studies utilizing monkeys, rats and mice, and confirmed a decrease in episodic memory and other cognitive functions with age across species (Simen et al. 2011). These changes are believed to be associated with, but not limited to, age-dependent neuroinflammation (Barrientos et al. 2009; Gemma et al. 2005; Wan et al. 2007), demyelination (Vanguilder et al. 2012), impairment of synaptic receptors (Pagnussat et al. 2015), and increase of Ca^{2+} in the hippocampus (Foster 2012). Such a diverse spectrum of age-related pathology will require much more work to understand any compounding effects of r-mTBI in the elderly. Nevertheless, our model demonstrates a clear TBI-dependent neuroinflammatory response, and although we do not observe increased tau phosphorylation or increased cognitive deficits following TBI, nilvadipine treatment resulted in significant improvement in all three of these domains. Nilvadipine significantly decreased neuroinflammation in the corpus callosum and cortex, as shown by lower

levels of astrogliosis and microgliosis. Moreover, psyk accumulation was colocalized with activated microglia in the corpus callosum in the 5r-mTBI-vehicle mice, and nilvadipine treatment reduced both, psyk and Iba1 signals, to the sham levels. The neurobehavioral deficits in the mice correlate with tau phosphorylation, but nilvadipine did not have a significant effect on cognitive performance in the sham mice, which exhibited similarly high levels of tau phosphorylation. Thus, it seems that the inflamed post-r-mTBI environment was necessary for nilvadipine to exhibit therapeutic properties. As mentioned previously, the spleen tyrosine kinase regulates neuroinflammation through NFkB activity, and also phosphorylates tau directly at Tyr18 (Paris et al. 2014). Nilvadipine inhibits syk, and this also activates PKA which phosphorylates Ser9 of GSK3 β , thereby inactivating this potent tau kinase. The syk inhibitory properties of nilvadipine have been previously demonstrated in mouse models of AD and tauopathies (Paris et al. 2014), but more work is required to confirm that this is the mechanism through which it elicits the favorable responses described in this study. Nilvadipine is also an L-type Ca²⁺ channel blocker (Peters et al. 2015) and similar compounds have been investigated in TBI clinical trials (albeit these were more severe TBI cases) with very confounding results (G.-Z. Xu et al. 2013). We have previously shown efficacy of the non-blood-pressure-lowering enantiomer of nilvadipine ((-)-nilvadipine) in the controlled cortical impact mouse model of TBI (Ferguson, pers. comm). Given the positive results from nilvadipine treatment in this study in aged mice, determining whether or not this enantiomer has potency in this mild TBI model was among the studies we next planned, in order to elucidate the mechanism of action and pursue nilvadipine's potential as a therapeutic for repetitive mild traumatic brain injury. In addition, we of course wanted to repeat the study with younger hTau mice, not only to address another age group at significant risk for TBI in humans (young adults) but also to determine the role of aging

in r-mTBI pathology and treatment. Chapter 3 details the effects of both nilvadipine treatment and (-)-nilvadipine treatment in an identical study carried out in 2-3 month old hTau mice.

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Chapter 3 – Investigation of the effect of treatment with nilvadipine and selective syk inhibitor in the young hTau mice following r-mTBI

3.1 Introduction

In the previous chapter we described a study in which 24-26-month old hTau mice were exposed to 5r-mTBI or 5r-sham, followed by a 3-week treatment with the drug nilvadipine (or vehicle control). Nilvadipine attenuated inflammation, tau phosphorylation and cognitive decline in aged hTau mice (Morin et al. 2018). Our data suggest that nilvadipine may represent a potential drug for TBI, however further work was required to address the effects of nilvadipine in young mice and to investigate the contributory role of the antihypertensive (calcium channel blocking) properties of nilvadipine. Below, we discuss the importance of these aspects and introduce an additional study to overcome these limitations.

3.1.1 TBI in the aged and young brain

Evidence of age effect on TBI outcomes in humans

Multiple clinical and preclinical studies have shown that age influences TBI pathology and that the same severity of injury may lead to different outcomes in the young and the elderly. For example, one study in Taiwan analyzed medical records of 2081 adults (aged 20-64 or >65 years old) diagnosed with moderate-severe TBI between 2009 and 2016, and found that older age was associated with higher GCS among individuals who received similar types of injuries (Rau et al. 2017). Several clinical studies (listed below) compared pathological markers of TBI, such as S100 β , GFAP and tau in populations of different ages and reported a stronger signal for

these markers in older individuals versus in comparably injured younger individuals. One such study conducted a gene profiling of blood in TBI victims younger than 35 or over 60 years old at the time of injury and showed an increase in calcium binding proteins and a decrease in growth factors, both linked to poor recovery, in the older but not younger individuals (Cho et al. 2016). Another study demonstrated a significant increase of S100 β in plasma within 3 h after mTBI in individuals >65 years old but not in their younger counterparts <65 years old (Calcagnile et al. 2013). These studies highlight that the aged brain appears to have a higher vulnerability to TBI which results in a more severe response than would occur for the same level of injury in a younger brain. However, a separate study by Gardner and colleagues recently suggested that strong age-related pathology in a healthy aged brain may mask TBI effects (Gardner et al. 2018). In this study, plasma levels of p-tau, total tau and GFAP were evaluated in mTBI patients and controls and stratified into three different age groups based on the age at injury (< 40, 40-60, >60 years old). The results indicated that p-tau and GFAP were able to discriminate between mTBI and control in the young and middle-aged groups, but they had a decreasing accuracy in differentiating mTBI from control groups in the >60 cohort. The authors also reported that healthy individuals showed age-dependent increases in p-tau, but not in GFAP, which may be due to normal aging (patients with dementia were excluded from the enrollment, hence p-tau baseline signal was less likely to be associated with neurodegenerative conditions). This observation is supported by our previous study of 24-26-months old r-mTBI mice where baseline levels of p-tau were high enough to mask TBI effect (Morin et al. 2018).

Evidence of an age effect in animal models of TBI

Several animal models of mTBI have been developed to study the pathological and behavioral consequences of TBI, but only a few have addressed age-related variables such as

chronological age, age at injury and time since injury (Calcagnile et al., 2013; Cheng et al., 2018; Ferguson et al., 2016; Mouzon et al., 2018). To date, the preclinical studies that address how age at injury influences mTBI provide contradictory results. Cheng et al. compared mice (WT and PSAPP) that received two mTBI using CHIMERA at the age of 6 or 13 months (Cheng et al. 2018). Age at injury in both WT and PSAPP mice did not alter behavioral response 14 days post mTBI. Inflammation was increased in old WT mice after injury but decreased in old PSAPP mice compared to their young counterparts. Axonal injury was found in both strains of mice at young age but not in aged animals. Another study compared WT mice subjected to r-mTBI at 5 weeks or 4 months of age and found that r-mTBI exacerbated cognitive functions and inflammation in a similar manner between the groups (Mannix et al. 2017). In our previous work, using the 5r-mTBI model, we explored the influence of age at injury (young [3 months] and aged [12 months]) on behavioral and pathological outcomes in hTau mice (Ferguson et al., 2017; Mouzon et al., 2018). At 24h and 15d post injury, the TBI-dependent increases of Iba1, GFAP and p-tau (RZ3, CP13) were similar between the young and old mice. However, the younger animals showed greater APP signal in TBI vs controls at 24 h compared to aged mice, while the aged animals had a higher phospho-tau at Ser396/404 (PHF-1) at 15 days. Interestingly, young mice (both males and females) exhibited deficits in spatial memory but not in motor coordination. In the aged group, female, but not male, mice showed TBI-induced motor and memory impairments, indicating possible age-dependent effect of sex on outcomes in this model of TBI. Most studies that have explored age as a variable have provided evidence that TBI induced detrimental changes in older rodents when compared to their young counterparts.

3.1.2 Hypotensive properties of nilvadipine

Previously, we did not account for the potential effect of the alternative mechanisms targeted by nilvadipine, namely its calcium channel blocking potential (hence its use as an antihypertensive drug) (Peters et al.2015). In one of our former studies, we treated 13-month old Tg APPsw mice, which mimic AD-like pathology including reduced cerebral blood flow (CBF), with nilvadipine (1mg/kg) daily for 15 days, and they demonstrated an increase in CBF compared to the vehicle treated mice (Paris et al. 2004). Thus, the positive effects of nilvadipine treatment could, at least in part, result from its cerebrovascular influence and resulting increased cerebral blood flow. Interestingly, nilvadipine is a racemate, and the vasoactive properties are due to the (+) enantiomer, with the (-) enantiomer showing no vasoactivity (Tokuma and Noguchi 1995; Paris, Ait-Ghezala, et al. 2014). In a previous study, (-)-nilvadipine administered to transgenic P301S mice for 4 consecutive days inhibited syk phosphorylation, decreased p-tau and A β pathology, and resolved cognitive deficits (Paris, Ait-Ghezala, et al. 2014). Therefore, in these current studies, having seen the positive effects of the racemate in aged mice with TBI (Chapter 2), we also included treatment with the non-vasoactive (-)-nilvadipine enantiomer (internal reference name ARC031) to investigate whether treatment effects on TBI outcomes required the vascular effects of racemic nilvadipine.

3.1.3 Hypothesis

- 1) 5r-mTBI will cause more distinctive cognitive and pathological impairments in the young mice, compared to the aged mice described in chapter 2, due to the lack of age-driven deteriorations which may have masked the response to injury.
- 2) Nilvadipine administered in the young 5r-mTBI mice for 21 days (recapitulating the treatment paradigm in Chapter 2) will attenuate injury-dependent behavioral and pathological changes.
- 3) Treatment with non-vasoactive (-)-nilvadipine will mitigate TBI-induced outcomes in a similar manner as the racemic nilvadipine due to the inhibition of syk.

3.2 Methods and Materials

3.2.1 Animals

Male and female hTau mice 12-14 weeks old (weight 19-25g) were sourced from Jackson Laboratories (Bar Harbor, ME). The animals were housed under standard laboratory conditions (14-hour light/10-hour dark cycle, $23\pm 1^{\circ}\text{C}$, $50\pm 5\%$ humidity) with free access to food and water. All procedures were carried out under Institutional Animal Care and Use Committee (IACUC) approval and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2.2 Experimental groups and study design

A total of 72 mice were randomly assigned to 1 of 6 groups ($n=12$ per group): repetitive sham/vehicle, repetitive sham/nilvadipine, repetitive sham/ARC031, repetitive injury/vehicle, repetitive injury/nilvadipine and repetitive injury/ARC031. The r-mTBI (total of 5 hits with an inter-concussion interval of 48h) was administered to mice as previously described in Chapter 2. Sham injured animals (5 anesthesia, 48h apart) underwent the same procedures and were exposed to anesthesia for the same length of time as the mTBI animals. Either nilvadipine, ARC031 or vehicle (PBS:PEG 1:1) were injected intraperitoneally ($100\ \mu\text{l}$, i.p.) daily for 21 days, with the first injection administered immediately following the last injury. The behavior analysis began 24h after the last mTBI/anesthesia for each group, as shown in Figure 7. Euthanasia was performed 22 days after the last mTBI/sham procedure. Researchers were blind to animal group assignments during both neurobehavioral experiments and immunohistochemistry. Data from the previous study (Morin et al. 2018), where aged mice (24-26 months old) underwent the same procedures, were used to compare the effect of age.

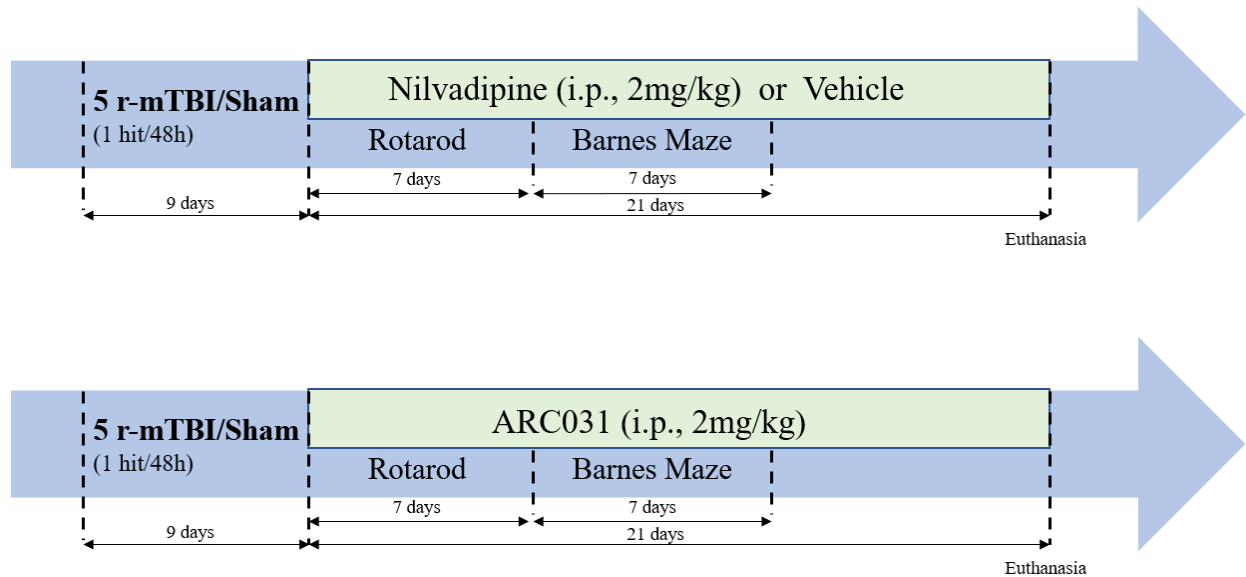


Figure 7. Study design

3.2.3 Injury Protocol

R-mTBI injury was performed as previously described (Mouzon et al. 2012, 2014; Tzekov et al. 2014, 2016; Ojo et al. 2015). All animals were anesthetized with 1.5 L/min of oxygen and 3% isoflurane prior to r-mTBI or sham injury. The heads were shaved and mice were placed on a heating pad to maintain body temperature at 37°C to prevent hypothermia. The head of each animal was fixed in a stereotaxic frame, and the blunt impactor tip (3 mm diameter) was positioned midway to the sagittal suture. The injury was triggered at 5 m/s velocity and 1.0 mm depth, with a dwell time of 200 milliseconds, using a myNeuroLab controller device (Impact One™ Stereotaxic Impactor, Richmond, IL). All mice experienced short-term apnea (<20 sec) and showed no skull fractures. All animals were allowed to recover from anesthesia on a heating pad and then returned to their cages with water and soft food access. Sham animals received

anesthesia alone for the same duration of time as the r-mTBI mice, to control for the effects of repeated anesthesia. Mice were monitored daily for any abnormalities in behavior.

3.2.4 Treatment

All mice received either nilvadipine, ARC031 or vehicle via i.p. injections for 21 days, starting immediately after the last injury/sham (the first injection was administered while animals were still under anesthesia). The treated groups received 2mg/kg nilvadipine (equivalent to human dose 8 mg conferring antihypertensive activity) or 2mg/kg ARC031 dissolved in a 1:1 solution of PBS and PEG (polyethylene glycol) vehicle solution (Paris, Ait-Ghezala, et al. 2014). The animal equivalent dose (AED) was calculated using a conversion protocol based on the exponent of the body surface area (K_m) with account for differences in metabolic rate: $AED (mg/kg) = Human\ dose (mg/kg) \times K_m\ ratio = 8\ mg/60kg \times 12.3 \approx 2\ mg/kg$ (Nair and Jacob 2016). The injection volume (100 μ l) was calculated based on the average animal's weight (0.028kg). Untreated animals underwent the same procedure, but received vehicle solution only (PBS:PEG/1:1). All solutions were freshly prepared every day before the injections.

3.2.5 Motor and cognitive functions assessment

Motor function was assessed using the Rotarod apparatus by monitoring the time a mouse could remain on an accelerating rod in the same manner we described previously in Chapter 2. Learning and spatial memory were evaluated by monitoring the distance and time taken to find an escape hole over a period of 6 training day (learning) and a final probe trial (memory) in the same manner we described in Chapter 2.

3.2.6 Tissue collection and processing

Twenty-two days after their last injury/anesthesia all animals were anesthetized with 3% isoflurane and perfused transcardially with phosphate-buffered saline (PBS), pH-7.4. For each mouse, after perfusion, one hemisphere was frozen and the other was blocked and processed as previously described in Chapter 2.

3.2.7 Immunohistochemistry

Immunohistochemistry for GFAP, Iba1 was carried out as described in Chapter 2. Imaging of non-fluorescent samples stained for GFAP/Iba1 was performed on an Olympus DP72 microscope at 10x magnification.

3.2.8 Fluorescent staining

GFAP/Iba1/RZ3/psyk: Fluorescent staining was performed as described in Chapter 2 using the following antibodies: Iba1 (ab107159, Abcam), GFAP (7857983, Aves Labs), phosphorylated tau at Thr231 (RZ3) (generously provided by Dr. Peter Davis, The Feinstein Institute for Medical Research, Bronx, NY) and phosphorylated spleen tyrosine kinase psyk (Tyr525/526) (2710S, Cell Signaling). Fluorescent imaging was performed using a confocal microscope (LSM 800 Zeiss) at 63x magnification. Z-stacks were recorded for every image and orthogonal projections were obtained to enable a 3D representation of the picture. (For immunofluorescent comparison between the young and aged mice, we used samples from the older animals described in Chapter 2).

3.2.9 Immunohistochemical quantification

Immunoreactivity for cell markers was measured by quantitative optical segmentation as previously described in Chapter 2. Three nonoverlapping regions of interest (ROI) of 100 μm^2 per image were then selected for the hippocampus, the body of the corpus callosum and the cortex. ROI were selected in the similar regions of the analyzed brain areas between the samples regardless of the obvious presence or absence of the pathological signal. A coverage area (%) per ROI was calculated and the mean value for each animal was used for further statistical analysis.

3.2.10 MesoScale Discovery (MSD) Multi-Spot Assay

A cytokine profile was assessed in cortical mouse homogenates using the MSD Proinflammatory Panel 1 (mouse) kit (V-PLEX K15048D). Cortical homogenates were prepared by adding 500 μl of M-PER solution which contained 1% EDTA and 1% of protease/phosphatase inhibitors cocktail. Then, samples were sonicated and centrifuged at 10,000 rpm (4°C) for 10 min and the supernatant was separated for further analysis. First, the calibration solution Diluent41 was prepared according to the kit protocol. Samples and controls were diluted 2-fold in Diluent41. Then, a combined detection antibody solution was prepared by mixing each antibody 5-fold with Diluent45. The plate was thoroughly washed 3 times with Wash Buffer and loaded with samples (50 μL /well) followed by a 2 h incubation at the room temperature. Then, the plate was washed 3 times and detection antibody solution was added to samples at 25 μL /well. After a 2 h incubation, the plate was washed again with Wash Buffer and 2X Read Buffer T was added at a concentration 150 μL /well followed by the plate analysis on the MSD instrument (MESO Quick Plex SQ120).

A Tau profile was assessed using a similar MSD protocol using the Phospho (Thr231)/Total Tau kit (V-PLEX K15121D) following manufacturer's instructions. Briefly, the plate was blocked with Blocker A for 1h followed by adding the samples and calibrators into the wells. After 1h of incubation, the plate was incubated for 1h with detection antibodies and the Read Buffer T was added to analyze the plate. The plate was washed with the Wash Buffer after each step.

(Both cytokine and tau analyses, performed by the MSD platform, included the cortical tissue from the aged mice (24-26 months old) from the previous study described in Chapter 2, on which MSD ELISA had not previously been performed. Samples from young and aged mice underwent MSD protocol manipulations at the same time).

3.2.11 Statistical analysis

All experimental data were analyzed using JMP 12 and PRISM Software. The data were checked for normality using Skewness-Kurtosis and Goodness of Fit. If normal, parametric methods (t-test and one-way ANOVA) were applied to calculate the significance between different experimental groups ($p < 0.05$ were considered significant). The Shapiro-Wilk test was used if data were not normally distributed. All data were transformed to logarithm or square root, when required, to reach normality before further analysis. Repeated-measure analysis of variance (MANOVA) was used to analyze continuous performance of mice in the Barnes Maze and Rotarod ($p < 0.05$ is significant). Error bars represent the standard error of the mean.

3.3 Results

3.3.1 Motor assessment

Motor functions were assessed using Rotarod on days 1, 3, 5 and 7 post-last TBI. Overall, all mice except the r-mTBI-vehicle group exhibited at least 40% increase in latency to fall over a 7-day period (Fig.8 A). R-mTBI-vehicle mice did not demonstrate improvement in their performance when compared to the baseline. Nilvadipine treatment in the injured mice ameliorated their motor deficits to levels not significantly different from sham mice, without affecting the behavior in healthy sham mice (r-mTBI-vehicle vs sham-vehicle $p < 0.0001$, r-mTBI-nilvadipine vs r-mTBI-vehicle $p < 0.001$, sham-nilvadipine vs sham-vehicle $p > 0.05$, r-mTBI-nilvadipine vs sham-vehicle $p > 0.05$, MANOVA). Treatment with ARC031 did not show any therapeutic effect in the r-mTBI mice (Fig.8 B, $p > 0.05$, MANOVA).

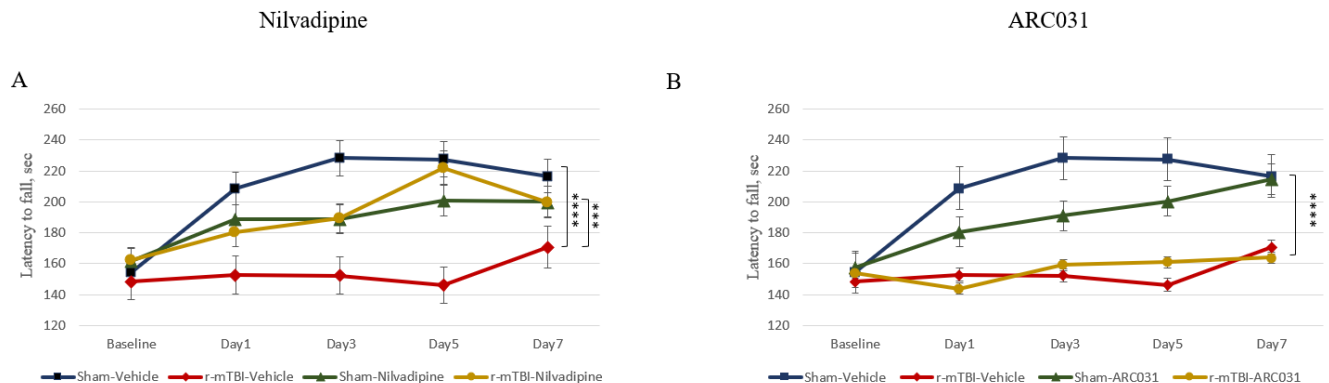


Figure 8. Evaluation of the effect of nilvadipine or ARC031 on motor performance. (A,B) Mice in the r-mTBI-vehicle group showed a significant decrease in their performance compared to the sham-vehicle ($p < 0.001$, MANOVA). (A) Treatment with nilvadipine ameliorated motor deficits in the r-mTBI mice (r-mTBI-Nilvadipine vs r-mTBI-vehicle, $p < 0.001$, MANOVA). (B) In r-mTBI mice treated with ARC031, no differences were observed in the latencies compared to the r-mTBI-vehicle ($p > 0.05$, MANOVA).

3.3.2 Memory assessment

Short-term memory was assessed using Barnes Maze during days 8-14 post last TBI and the probe trial was recorded on day 15. Sham-vehicle mice demonstrated a 83% decrease of the cumulative distance and a 71% decrease in the distance traveled from day 1 to 6 (Fig.9 A, B). R-mTBI vehicle mice exhibited a 25% reduction of the cumulative distance during days 1-3 which reached plateau on days 4-6 (r-mTBI-vehicle: day1 vs day 6 $p < 0.05$, one-way ANOVA). R-mTBI-vehicle mice had significantly higher cumulative distance (vs sham-vehicle $p < 0.001$, MANOVA) and distance traveled (vs sham-vehicle $p < 0.0001$, MANOVA) than controls. Travel distance in r-mTBI-vehicle mice stayed consistent with day 1 during an entire acquisition period (r-mTBI-vehicle: day 6 vs day 1 $p > 0.05$, one-way ANOVA). On days 2-6, r-mTBI-vehicle mice traveled longer time than sham-vehicle (r-mTBI-vehicle vs sham-vehicle: day2 $p < 0.01$, day3 $p < 0.01$, day4 $p < 0.001$, day5 $p < 0.001$, day6 $p < 0.001$, one-way ANOVA). Treatment with nilvadipine in r-mTBI mice lead to a 44% decrease in cumulative distance (r-mTBI-nilvadipine vs r-mTBI-vehicle $p < 0.01$, r-mTBI-nilvadipine vs sham-vehicle $p > 0.05$, MANOVA) and a 28.6% decrease in the distance traveled (r-mTBI-nilvadipine vs r-mTBI-vehicle $p < 0.0001$, r-mTBI-nilvadipine vs sham-vehicle $p < 0.01$, MANOVA). Similarly, a decrease in the latency to enter the target box from day 1 to day 6 was observed for sham-vehicle (44%) and sham-nilvadipine (25%) groups (Fig.9 C). After r-mTBI, mice spent 50% longer time to locate the box than sham-vehicle, with only transient improvements on days 3-5. A 35% increase was recorded for the r-mTBI-nilvadipine mice compared to sham-vehicle, although notably improved when compared to the injured controls (20%). During the probe trial, 5r-mTBI-vehicle mice spent a significantly longer time locating the target hole indicating deficits in spatial memory (Fig. 10 D; r-mTBI-vehicle vs sham-vehicle $p < 0.05$).

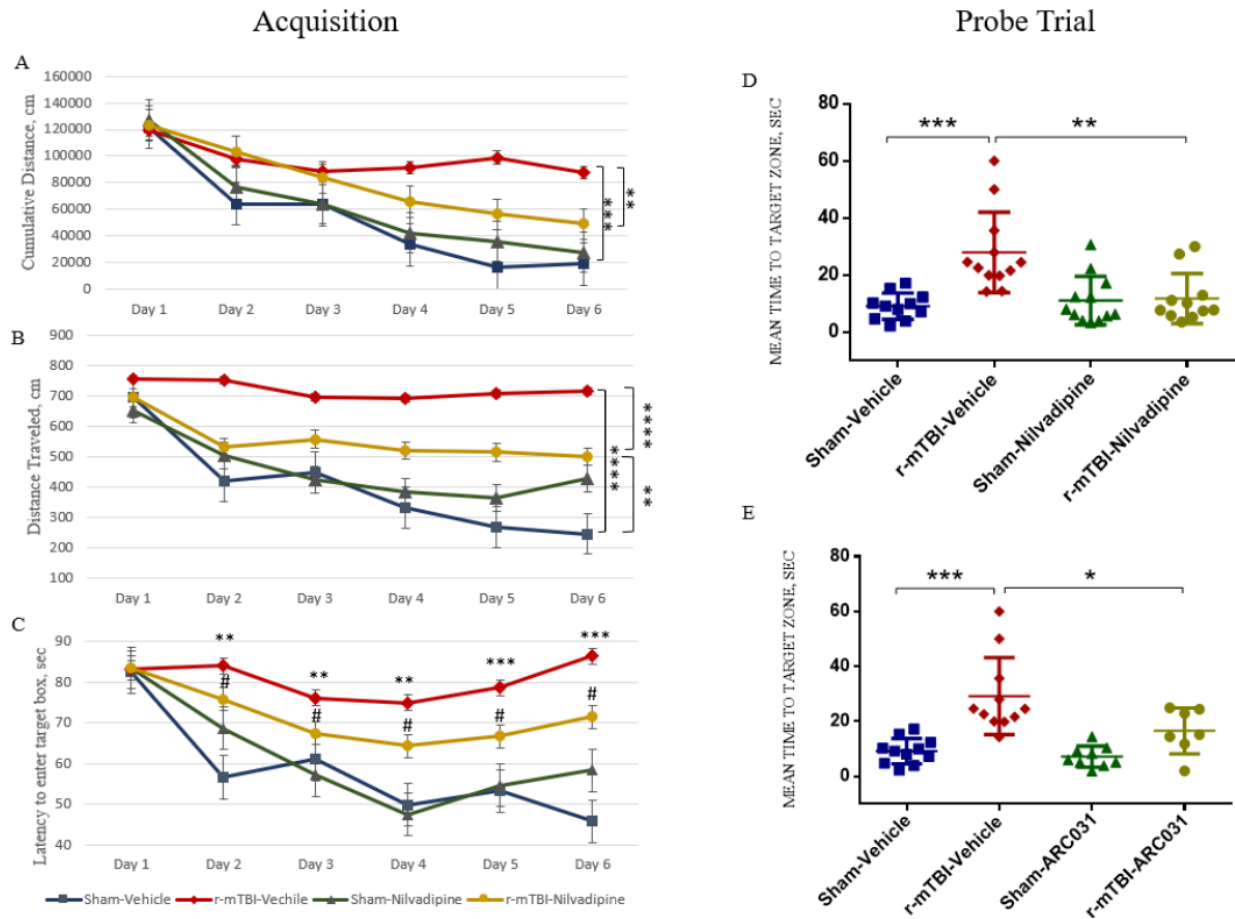


Figure 9. Evaluation of learning and memory. Compared to sham-vehicle, r-mTBI-vehicle mice exhibit learning deficits during the 6 days of acquisition. All injured groups travelled greater distance (A, $p < 0.001$; B, $p < 0.001$) and failed to enter the box (C, days 2-4, $p < 0.01$, days 5-6 $p < 0.001$) compared to sham-vehicle mice. R-mTBI-Nilvadipine mice showed an improvement in all 3 parameters compared to the r-mTBI-vehicle (cumulative distance $p < 0.01$, distance traveled $p < 0.0001$, latency to target box $p < 0.05$). (D) Probe data show that r-mTBI-vehicle mice spent more time to locate the target hole compared to the sham-vehicle controls ($p < 0.05$, one-way ANOVA). Nilvadipine treatment in the r-mTBI ameliorated memory functions compared to r-mTBI-vehicle ($p < 0.05$, one-way ANOVA). (E) In the r-mTBI-ARC031 mice, mean time to target hole decreased compared to the r-mTBI-vehicle during the probe trial ($p > 0.05$, one-way ANOVA).

Nilvadipine recovered these deficits back to the control levels with no effects on healthy sham mice (r-mTBI-nilvadipine vs r-mTBI-vehicle $p < 0.01$, r-mTBI-nilvadipine vs sham-vehicle $p > 0.05$, one-way ANOVA). ARC031 also decreased r-mTBI-induced memory impairments during the probe trial (Fig.9; r-mTBI-ARC031 vs r-mTBI-vehicle $p < 0.05$, r-mTBI-ARC031 vs sham-vehicle $p > 0.05$, one-way ANOVA).

A comparison of the behavior between the young and old sham mice, presented in the previous chapter, revealed a significant difference in Rotarod and BM performance among healthy controls (Fig.10). Motor evaluation indicated an increased baseline level of latency to fall in the young mice compared to the old, which sustained during a 7-day testing (Fig.10A; young sham-vehicle vs old sham-vehicle $p < 0.0001$, MANOVA). Spatial memory assessment demonstrated no difference between the young and old sham mice on day 1 for distance traveled, cumulative distance, and latency to enter the box (Fig.10B-D). However, throughout days 2-6, younger mice showed a significant improvement in locating the target box compared to day 1, while older mice showed little change overtime.

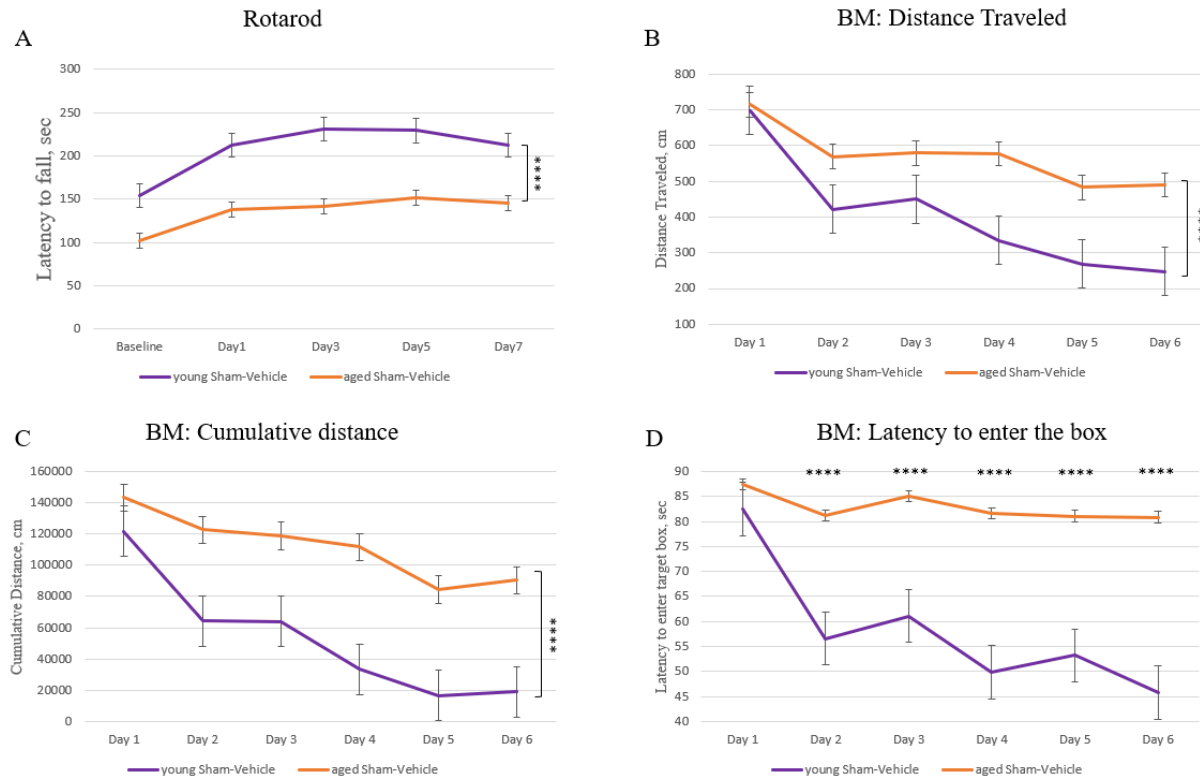


Figure 10. Age-dependent behavior deficits in sham-vehicle mice. A – 24-26-month old sham-vehicle mice spent significantly less time on the rotarod compared to young sham-vehicle ($p < 0.0001$). B-D – Barnes Maze acquisition data showed an increase in distance traveled ($p < 0.0001$), cumulative distance ($p < 0.0001$) and time to enter the box (days 2-6, $p < 0.0001$ for each day) in the aged sham-vehicle mice vs young sham-vehicle. Data analyzed using MANOVA (A,B,C) and one-way ANOVA (D).

3.3.3 Immunohistochemistry

GFAP staining revealed no gliosis in the healthy tissue of sham-vehicle mice (Fig.11 A). An increased astroglial activity was prominent in the r-mTBI-vehicle mice in the corpus callosum ($p < 0.05$, one-way ANOVA) and areas of cortex in surrounding injury site ($p < 0.001$, one-way ANOVA) (Fig.11 A-D). This increase was reduced by the nilvadipine treatment in all

three analyzed brain areas (corpus callosum $p < 0.01$, cortex $p < 0.05$, one-way ANOVA). In the r-mTBI-ARC031 mice, a significant decrease of astrogliosis was shown in corpus callosum ($p < 0.001$, one-way ANOVA) but not in cortex ($p > 0.05$, one-way ANOVA) (Fig.11 C-D). Immunofluorescent analysis demonstrated the extent of astroglial reaction in cortex of r-mTBI-vehicle mice spreading from the superficial into the deeper cortical layers (Fig.12).

Immunofluorescent analysis of GFAP in young and aged mice (samples from the mice described in Chapter 2, included to facilitate direct comparison) showed a TBI-induced increase in GFAP signal in hippocampus, corpus callosum and cortex across both age groups (Fig.13 A,B). Quantification analysis of fluorescent intensity of GFAP showed $> 50\%$ increase in hippocampus (Fig. 13 C, F; young: r-mTBI-vehicle vs sham-vehicle $p < 0.05$; aged: r-mTBI-vehicle vs sham-vehicle $p < 0.05$, one way ANOVA) and $> 60\%$ in cortex in both young and aged mice (Fig.13 D, G; young: r-mTBI-vehicle vs sham-vehicle $p < 0.001$; aged: r-mTBI-vehicle vs sham-vehicle $p < 0.01$, one way ANOVA). In the r-mTBI-nilvadipine mice, GFAP signal was decreased compared to the r-mTBI-vehicle (young: hippocampus $p < 0.05$, cortex $p < 0.05$; aged: hippocampus $p < 0.05$, cortex $p < 0.01$, one-way ANOVA). Similarly, a greater area % of GFAP was detected in r-mTBI-vehicle mice in the corpus callosum of both young ($p < 0.05$) and aged ($p < 0.001$) groups compared to their retrospective sham-vehicle mice (Fig.13 E, H). Treatment with nilvadipine decreased TBI-induced GFAP signal in corpus callosum in both groups (young: $p < 0.01$; aged: $p < 0.01$, one-way ANOVA).

IHC staining for Iba1 revealed a 50% increase in microgliosis in the r-mTBI-vehicle mice compared to sham-vehicle mice in the corpus callosum ($p < 0.01$, one-way ANOVA) but not in cortex ($p > 0.05$, one-way ANOVA) (Fig.14). In the r-mTBI-nilvadipine mice, Iba1 showed a 50% decrease (to close to sham levels) compared to the r-mTBI-vehicle mice ($p < 0.05$, one-way

ANOVA) (Fig.14 B). Similarly, treatment with ARC031 reduced Iba1 in corpus callosum of r-mTBI-ARC031 mice compared to r-mTBI-vehicle ($p<0.01$, one-way ANOVA) (Fig.14 B).

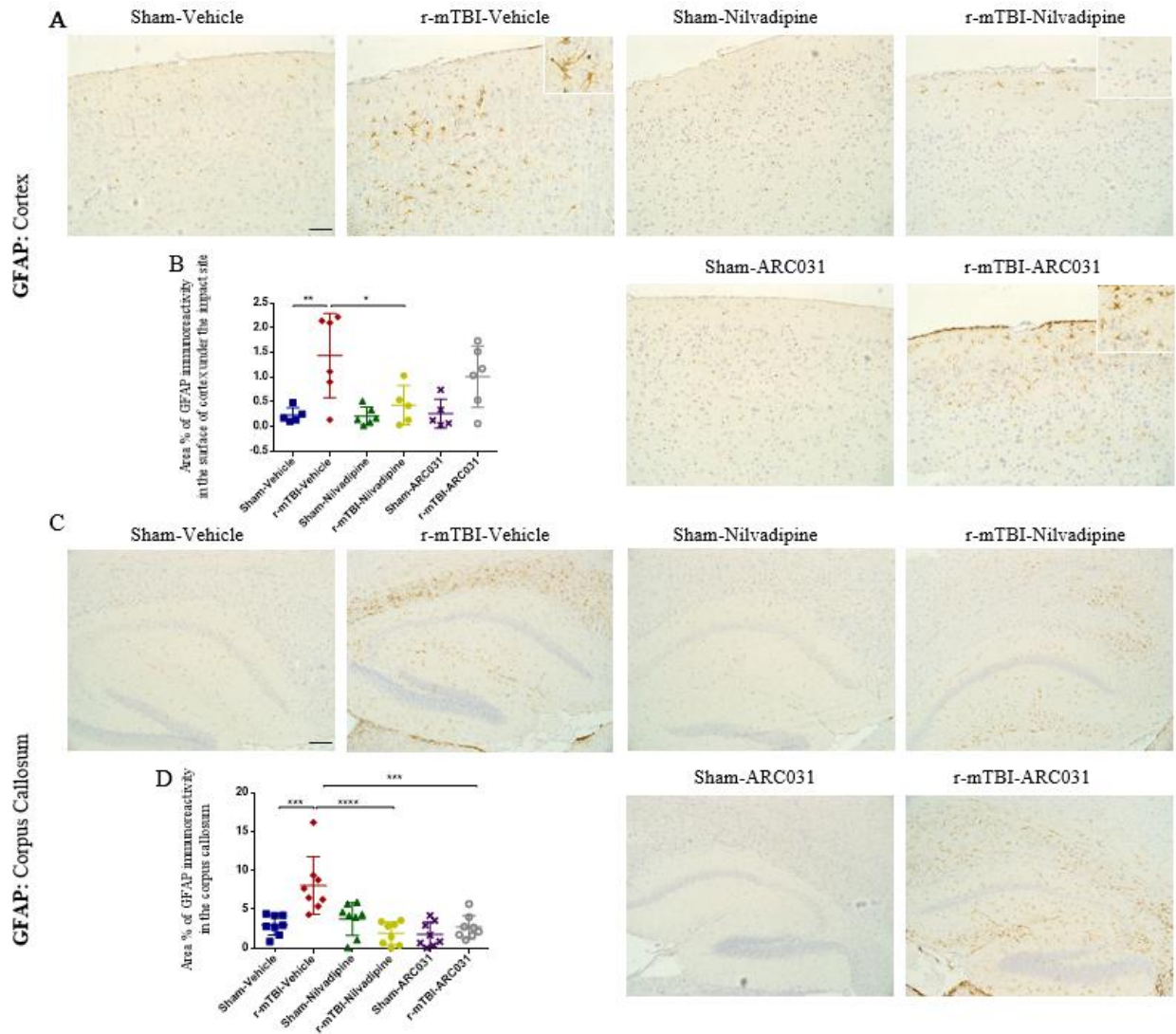


Figure 11. Evaluation of the effect of nilvadipine or ARC031 on astrocytes. (A-B) Representative images of GFAP in cortex. An increase in the area of GFAP staining was observed in the r-mTBI-vehicle mice compared to the sham-vehicle ($p<0.001$). This increase was suppressed in the r-mTBI-nilvadipine mice ($p<0.05$). (C-D) In the corpus callosum, r-mTBI-vehicle mice also exhibited elevated levels of GFAP compared to the sham-vehicle mice ($p<0.001$). Treatment with nilvadipine and ARC031 in the r-mTBI mice reduced astrogliosis ($p<0.0001$, $p<0.001$, respectively) compared to respective TBI-vehicle. Data are

presented as mean \pm standard error of the mean; significance was calculated using one-way ANOVA. Scale bars equal 100 μ m.

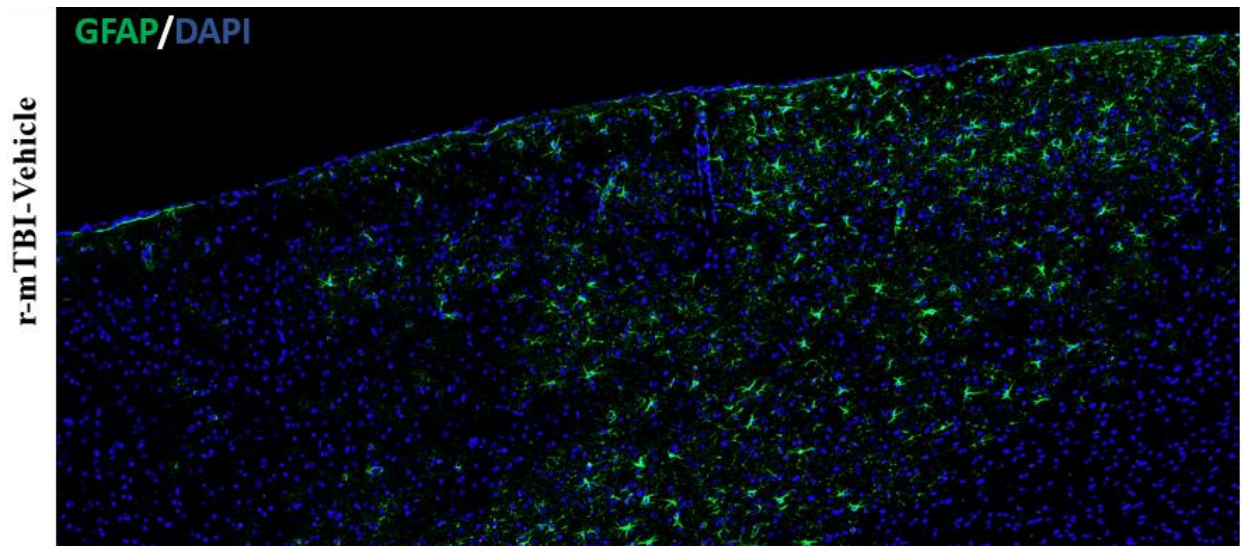


Figure 12. Immunofluorescent staining of **GFAP** in cortex. In the r-mTBI-vehicle mice, **GFAP** signal was found spreading from the surface of the cortex into the deeper layers. Scale bars equal 100 μ m.

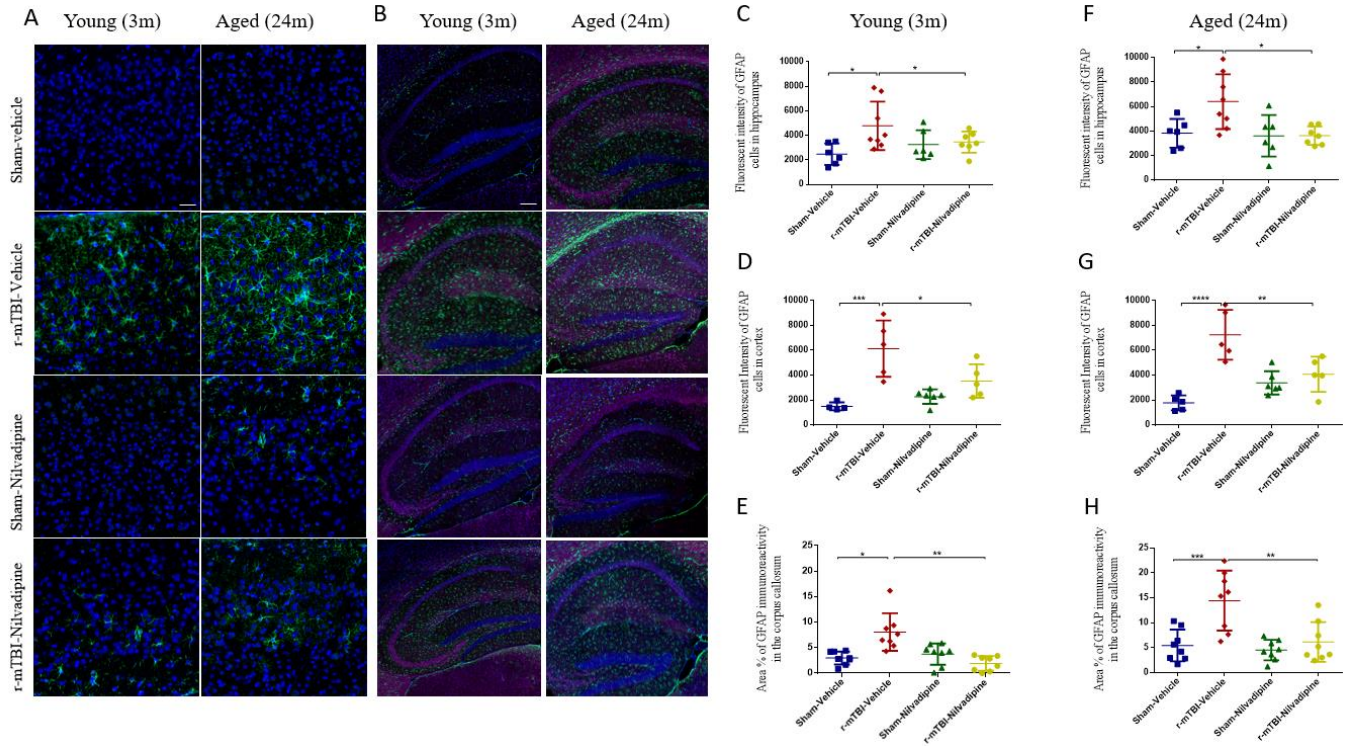


Figure 13. Immunofluorescence of GFAP and RZ3 in cortex, corpus callosum and hippocampus in the young (3 months) and aged (24 months) mice with r-mTBI. Representative images of cortex (A) show no differences in GFAP signal between the young and the old. In both groups (D,G), r-mTBI induced a significant increase in GFAP compared to the respective sham-vehicle (r-mTBI-vehicle vs sham-vehicle: young $p < 0.05$, aged $p < 0.05$). In the hippocampus (B), fluorescent intensity was increased after r-mTBI in both young (C, $p < 0.001$) and old (F, $p < 0.0001$) mice compared to sham-vehicle. Similarly, the area % was increased in the corpus callosum in the young (E, $p < 0.05$) and aged (H, $p < 0.001$) compared to their controls. Nilvadipine treatment decreased TBI-induced signal of GFAP in all areas for both young (cortex $p < 0.05$, hippocampus $p < 0.05$, CC $p < 0.01$) and aged (cortex $p < 0.01$, hippocampus $p < 0.05$, CC $p < 0.01$). No differences were found for RZ3 between the groups in all analyzed areas. All data were analyzed using one-way ANOVA. Scale bars equal 50μm (A) and 100μm (B).

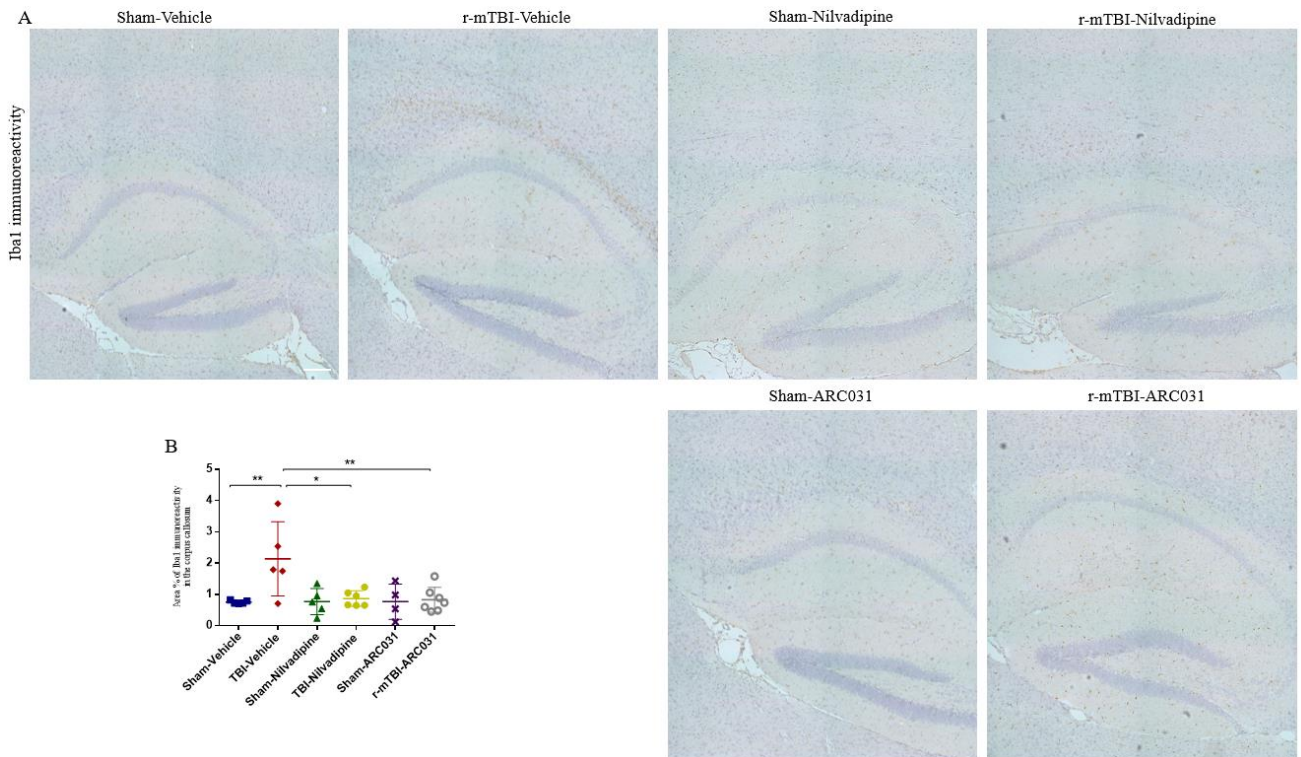


Figure 14. Evaluation of the effect of nilvadipine on microglia in the aged r-mTBI mice. A,B - after r-mTBI, Iba1 signal was increased in the CC compared to sham-vehicle ($p < 0.01$). A significant decrease of Iba1 was shown in the r-mTBI mice after treatment with nilvadipine ($p < 0.05$) and ARC031 ($p < 0.01$). Data was analyzed using one-way ANOVA. Scale bars equal $50\mu\text{m}$.

3.3.4 Inflammatory cytokines

A panel of proinflammatory cytokines was assessed in the cortices of young mice and compared with the cortical levels of the same cytokines in the aged brains from the previous study (Morin et al. 2018). Overall, the analysis revealed no differences in baseline levels of cytokines between the sham young and sham old mice (Fig.15 A-E). TBI-induced increases in selective cytokines were observed in the old (aged r-mTBI-vehicle vs aged sham-vehicle: KCGRO $p < 0.05$, $\text{TNF}\alpha$ $p < 0.05$, one-way ANOVA) but not young mice when compared to their

respective sham mice. Aged r-mTBI-vehicle mice also exhibited higher levels of KCGRO ($p<0.05$), TNF α ($p<0.01$) and IL-1 β ($p<0.001$) compared to young r-mTBI-vehicle (Fig.15 A-C). Accordingly, nilvadipine decreased abnormal cytokine levels in the r-mTBI aged mice to levels close to those observed in the age-matched sham mice (aged r-mTBI-nilvadipine vs aged r-mTBI-vehicle: TNF α $p<0.05$, IL-1 β $p<0.01$, IL-4 $p<0.01$, one-way ANOVA) but had no effect on the cytokine expression in young mice (Fig.15 A,C,D).

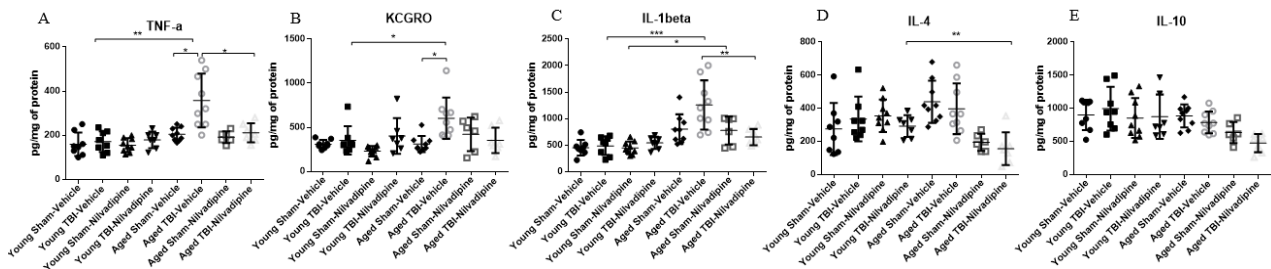


Figure 15. Quantitative analysis (pg/mg of protein) of proinflammatory cytokines in cortex of young and aged hTau mice. (A-B) Aged r-mTBI-vehicle mice expressed higher levels of TNF α ($p<0.001$) and KCRGO ($p<0.05$) compared to their young counterparts. Within the age group, r-mTBI-vehicle mice showed higher levels of TNF α ($p<0.05$) and KCGRO ($p<0.05$) vs sham-vehicle. (C) IL-1 β was overall increased in all aged mice compared to the young animals. Older r-mTBI-vehicle mice expressed higher levels of the cytokine compared to their young counterparts ($p<0.001$). For the aged mice, Nilvadipine decreased IL-1 β when compared to r-mTBI-vehicle group ($p<0.01$) (D) Nilvadipine had a stronger effect on the IL-4 reduction in the aged r-mTBI vs young r-mTBI mice ($p<0.01$). (E) IL-10 showed a trend in overall decrease for young mice compared to the aged. Data are presented as mean \pm standard error of the mean; significance was calculated using one-way ANOVA.

3.3.5 Tau phosphorylation

MSD ELISA analysis of cortical total tau and p-tau showed no TBI-induced changes in the young mice. The only significant difference observed was that sham-nilvadipine mice had significantly lower levels of p-tau compared to the r-mTBI vehicle mice ($p < 0.05$, one-way ANOVA). R-mTBI-nilvadipine were not significantly different from r-mTBI vehicle. A different trend was observed in the aged mice, where r-mTBI increased p-tau compared to sham mice ($p < 0.05$, one-way ANOVA). Treatment with nilvadipine attenuated tau phosphorylation in the aged mice ($p < 0.05$, one-way ANOVA). Total tau was slightly increased in all r-mTBI mice across different ages, however this increase was not significant for any comparisons of TBI vs respective sham (Fig.16). When normalized to total tau, p-tau did not differ between the groups within the same age (Fig.16 C).

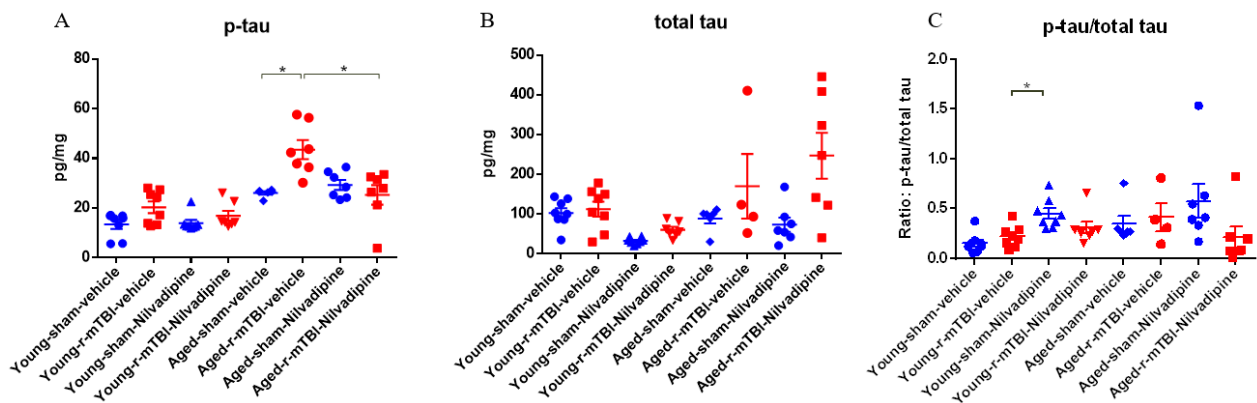


Figure 16. Quantitative analysis (pg/mg) of p-tau, total tau and p/tau/total tau in cortex of young and aged hTau mice. A - Quantitative analysis of cortical p-tau revealed no difference in sham-vehicle mice between young and old groups. Tau phosphorylation was increased in the aged r-mTBI-vehicle compared to the sham-vehicle mice ($p < 0.01$), whereas no significant differences were observed in young mice ($p > 0.05$). B - There was a trend in total tau increase in the young and aged r-mTBI-vehicle mice compared to their retrospective controls. C - The ratio of p-tau to total tau was not significant between the sham-vehicle/r-mTBI-vehicle and r-mTBI-vehicle/r-

mTBI-Nilvadipine mice in all age groups. In the young mice, tau ratio was significantly lower in the r-mTBI-vehicle mice compared to the sham-Nilvadipine ($p<0.05$) while in the aged mice there was no difference between the groups. Data are presented as mean \pm standard error of the mean; significance is calculated using one-way ANOVA.

3.3.6 Phosphorylated syk

Fluorescent signal for psyk was detected in the young r-mTBI-vehicle mice but absent in the young sham-vehicle mice (Fig.17). Patchy psyk distribution was found in the corpus callosum but it was not easily detected and could not be quantified. All detected areas of psyk accumulation were localized in proximity to Iba1-immunoreactive microglia. In the r-mTBI-nilvadipine and r-mTBI-ARC031 mice there was no evidence for psyk in the corpus callosum.

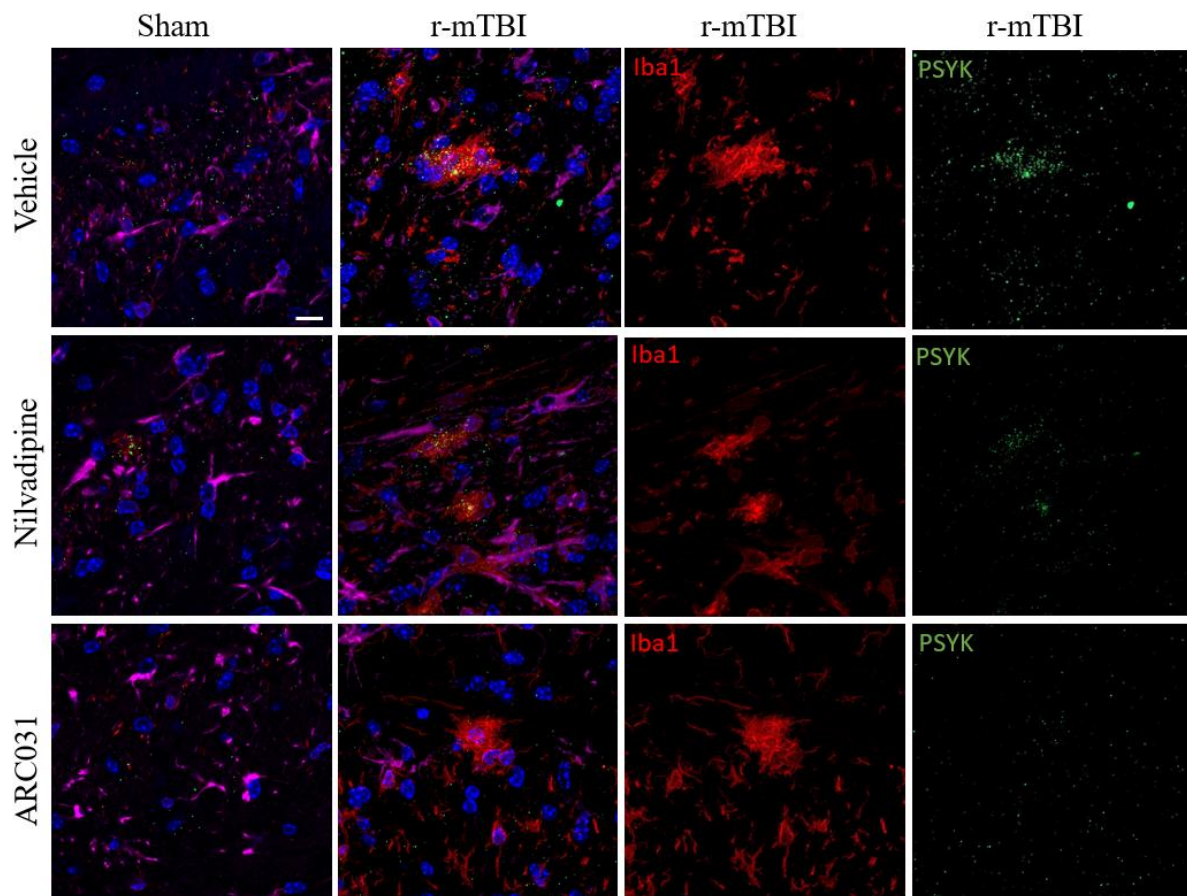


Figure 17. Evaluation of the effect of nilvadipine or ARC031 on **psyk**, **GFAP** and **Iba1** in corpus callosum. No sham animals showed reactive astrocytes, microgliosis or signs of psyk. In the r-mTBI-vehicle mice, microglia appear to acquire amoeboid morphology and colocalize with psyk accumulations. Treatment with nilvadipine or ARC031 eliminated signs of psyk in corpus callosum in the areas near Iba1-reactive microglia. Scale bars equal 20µm

3.4 Discussion

The work described in this chapter attempted to determine whether age at injury influences recovery in a preclinical model of mTBI by comparing the efficacy of nilvadipine in aged animals (seen in Chapter 2) with young animals investigated here.

We found that nilvadipine improved behavior and pathological impairments in young animals in a similar manner to the improvements observed in aged mice. Cognitive testing demonstrated a strong TBI-induced memory deficit in young vehicle-treated mice whereas none was seen in the aged r-mTBI-vehicle group during the probe trial on BM. When we compared the young and aged r-sham groups for both behavior tests, we found a decreased baseline in older mice versus the young. During motor assessment, healthy aged mice spent overall less time on the rotarod than young sham mice. In the BM, there was a decrease in short-term memory in older mice, suggesting that age adversely affects the baseline cognitive performance to such a degree as to mask any mTBI-induced cognitive deficits. Hence, in aged mice, Barnes maze testing could not differentiate whether r-mTBI exacerbated existing memory impairment.

Pathological analysis has confirmed the detrimental effect of r-mTBI in all age groups of mice. In particular, both young and old animals exhibited TBI-induced astrogliosis in the hippocampus, corpus callosum and cortex. When compared to our previous data in aged mice (Morin et al. 2018), baseline levels of astroglial density in the hippocampus and corpus callosum were lower in the healthy young mice versus their aged counterparts. Increased signal of immunoreactive microglia was also shown in young TBI mice in corpus callosum but not in cortex or hippocampus. Similar data were reported in aged TBI mice in our prior study (Morin et al. 2018). Moreover, we observed an increase in cortical levels of pro-inflammatory cytokine IL-1 β and a decrease of anti-inflammatory IL-10 in the aged sham mice versus young sham mice.

This suggests a greater level of neuroinflammation in aged animals, which may indicate a higher level of vulnerability to physical insults. Indeed, our r-mTBI model triggered a prominent inflammatory response in older animals as shown by the IHC analysis for GFAP. A greater density of reactive astrocytes was recorded for the aged r-mTBI-vehicle mice versus young counterparts in cortex under the injury site, hippocampus and corpus callosum. Molecular analysis of inflammatory cytokines in the cortex showed an increase in pro-inflammatory cytokines TNF α and KCGRO after r-mTBI in the aged but not in the young mice. Taken together, these data support the concept of age-driven neuroinflammation and an increased vulnerability of the aged brain to r-mTBI.

A significant effect of age on the ongoing pathology is supported by our findings of cortical and hippocampal age-specific tauopathy. When compared with our previous study in Chapter 2, aged sham mice had an increased expression of p-tau in the hippocampus and cortex when compared to their young sham counterparts. r-mTBI in young animals did not cause an increase in the cortical or hippocampal p-tau when compared to their respective sham. Molecular analysis of p-tau in the cortex also showed a lack of injury effect in the young mice, but a robust p-tau increase in old animals. In our earlier work, when we compared hTau mice that received 5r-mTBI at 3 or 12 months of age, we found a pronounced p-tau pathology in the aged but not young mice (Mouzon et al., 2018; Ferguson et al., 2017). Growing evidence suggest that tau hyperphosphorylation plays a major role in the chronic consequences of TBI, especially in the development of age-associated disorders, such as CTE, ARTAG and AD (Kulbe and Hall 2017; Petraglia et al. 2014; Kovacs et al. 2018; Tateno et al. 2015). Tau pathology observed in the aging brain was shown to be exacerbated by brain injuries and may be linked to stronger inflammation and cognitive deficits.

Our data revealed therapeutic efficacy of nilvadipine on pathological and cognitive outcomes indicating its potency as treatment for r-mTBI. It attenuated motor deficits in young but not old mice, which may arise from a more severe underlying pathology in older animals due to possible higher vulnerability to brain traumas. Nilvadipine was able to mitigate the memory deficits induced by r-mTBI in both age groups as shown by the BM. In both young and old r-mTBI mice, three-week treatment with nilvadipine reduced astrogliosis and microgliosis to the age-matched sham levels. This decrease was observed for all analyzed areas – cortex, hippocampus, corpus callosum (for astroglia) and corpus callosum only (for microglia). Molecular analysis of cortical levels of inflammatory cytokines showed a nilvadipine-induced decrease of TNF α and IL-1 β in the aged mice. We have previously demonstrated that nilvadipine exhibits therapeutic properties by inhibiting syk phosphorylation (Paris et al., 2014). In our previous studies, we showed that psyk is linked to neuroinflammation, tau phosphorylation and cognitive deficits. We also showed that r-mTBI caused psyk accumulation in the corpus callosum of aged mice (Morin et al., 2018). Notably, psyk patches were always colocalized with amoeboid microglia indicating a strong link with neuroinflammation. Nilvadipine was able to reduce psyk in mouse models of AD and aged r-mTBI (Morin et al. 2018; Paris et al. 2014). In the current study, we observed a TBI-induced increase of psyk in the proximity to microglia in the corpus callosum but to a lesser extent than in the old mice. These data suggest an age-dependent increase of psyk in the post-concussive brain. Nilvadipine inhibited syk phosphorylation in the young r-mTBI mice.

Treatment with ARC031 showed a slightly different effect in the r-mTBI mice compared to nilvadipine. First, it did not attenuate motor deficits, but it did improve memory impairment in the young mice. Second, it reduced astrogliosis in the corpus callosum but, unlike nilvadipine,

had no effect on reactive astrocytes in the cortex. It did, however, decrease microgliosis in the corpus callosum of young r-mTBI mice. Third, it decreased syk phosphorylation in the corpus callosum in a similar manner to nilvadipine. The lack of effect of ARC031 (compared to nilvadipine) on motor outcomes may be linked to the effects of nilvadipine on cerebral blood flow; possibly the effects on cortical astrogliosis may also be related to vascular involvement of nilvadipine that are absent in ARC031. Further studies will require the use of other anti-hypertensive calcium channel blockers in our 5r-mTBI model to validate this hypothesis.

The advantage of this study is the ability to compare injuries with the same TBI severity across different age groups. Overall, our findings demonstrate an age-dependent response to r-mTBI which is likely due to the age-driven pathological mechanisms in a healthy brain. Nilvadipine exhibited therapeutic efficacy in both young and aged mice and is proposed to act through the inhibition of syk, a potential target for both TBI and AD. However, the diversity of pathological and cognitive analyses suggests complex nature of the “age-TBI-treatment” interaction and requires further investigation. Our data provide valuable insights into the role of age of patients for treatment development and the necessity for considering different strategies to mitigate r-mTBI outcomes in young and old populations.

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Chapter 4 – Investigation of delayed treatment with anatabine in two different injury paradigms of r-mTBI

4.1 Introduction

In Chapter 1, we described the many faces of TBI pathobiology that vary from acute neuronal damage to long-lasting neurodegeneration. Of all aspects of TBI pathobiology, neuroinflammation is perhaps the most consistent, reported in most human studies and all preclinical models of TBI. In humans, markers of inflammation such as S100 β (Cho et al. 2016; Zongo et al. 2012), GFAP (Bogoslovsky et al. 2017) and cytokines IL-6/IL-1 β /TNF α (Devoto et al. 2017; Gill et al. 2018; Kanefsky et al. 2019) have been found in plasma in response to different types of TBI and at acute and chronic timepoints. Autopsy examination of brains with chronic TBI or CTE also confirm a profound role of long-lasting inflammation in neurodegeneration (Gentleman et al. 2004; Johnson et al. 2013; Kiernan et al. 2015; McKee et al. 2016). Nevertheless, existing drugs with anti-inflammatory action (e.g. progesterone (Stein 2015), statins (Robertson et al. 2017), minocycline (J. Meythaler et al. 2019)) fail to resolve TBI-associated neurological outcomes in clinical trials. Moreover, whether they actually have an effect on inflammation in the brain remains unclear, mainly due to the lack of effective real-time techniques that are able to measure neuroinflammation in a living person. A recent preclinical study has developed positron emission tomography (PET) markers to visualize microglia in mouse models of AD and EAE and demonstrated high purity of the signal and biological safety (Horti et al. 2019), however, human microglial imaging is still experimental (Largeau et al. 2017). Plasma biomarkers offer an alternative way to measure neuroinflammation, but they rather serve as surrogates and not a first-hand presentation of pathology. Here, we decided to test

a potent anti-inflammatory drug anatabine, which we previously showed to be effective in our models of AD (Verma et al. 2015; Paris et al. 2013) and EAE (Paris et al. 2013), and which showed promise for TBI treatment in an earlier study (S. Ferguson et al. 2016).

4.1.1 Anatabine

Anatabine is a natural alkaloid of the *Solanaceae* family which includes tobacco, potatoes, tomatoes and others. It was marketed in the United States by Rock Creek Pharmaceuticals as a dietary supplement and was used to treat joint pain and arthritis (Lanier et al., 2013). Later, anatabine was shown effective against thyroiditis, an inflammation of the thyroid, as assessed by the decrease of serum levels of thyroglobulin antibodies when administered 3 times daily for 3 months (Schmeltz et al. 2014). Preclinical models of thyroiditis in mice treated with anatabine also revealed a resolution of inflammation and a decrease of cytokines IL-1 β and IL-12 (Caturegli et al. 2012). Anatabine is a natural agonist of nicotinic receptors that binds the $\alpha 7$ subtype of nicotinic acetylcholine receptors (nAChR) (Levin et al. 2014). The large distribution of $\alpha 7$ nAChRs in the hippocampus makes it an attractive target for memory and learning deficits.

Our team demonstrated the potent anti-inflammatory properties of anatabine through the inhibition of signal transducer and activation of transcription-3 (STAT3) and NF κ B activation (Paris et al. 2013) (Fig.18). Similar to NF κ B, phosphorylated STAT3 translocates to the nucleus where it acts as a transcriptional factor increasing the expression of GFAP and pro-inflammatory cytokines (Oliva et al. 2012; Dawn et al. 2004; Yi et al. 2007). Increased levels of P-STAT3 and associated cytokines production were shown in rodent models of AD (Wan et al. 2010), ischemia (De Butte-Smith et al. 2012) and TBI (Oliva et al. 2012). Anatabine was able to not only reduce phosphorylation of STAT3 and suppress pro-inflammatory cytokines but also to decrease

phosphorylation of tau at multiple epitopes (RZ3, PHF-1, CP13) in Tg APP^{sw} and P301S mouse models of AD (Paris et al. 2013; Verma et al. 2015). In the PSAPP mice, treatment with anatabine for 6 months also reduced amyloid burden in the hippocampus and cortex (Verma et al. 2015). Similar therapeutic properties of anatabine were shown in mice with experimental autoimmune encephalomyelitis (EAE) modeling Multiple Sclerosis (MS), where a 16-day treatment decreased microgliosis and astrogliosis, suppressed STAT3 and NFκB phosphorylation and prevented demyelination in the spinal cord (Paris et al. 2013). In *in vivo* models of LPS-induced inflammation, anatabine reduced production of inflammatory cytokines IL-6, IL-1β and TNFα. Due to the powerful anti-inflammatory properties of anatabine, and the very strong neuroinflammatory presentation in our 5-hit mTBI mouse model (Mouzon et al. 2012, 2018) we explored the therapeutic potential of anatabine treatment in this model (5r-mTBI; 48 hours inter-injury interval). Wild type mice were treated with anatabine in their drinking water for 9 months, starting either immediately after the last injury/sham procedure or at 9 months after the last TBI/sham injury in a crossover design (Ferguson et al. 2016). Anatabine improved cognitive deficits at 6 months and decreased inflammation at 9 months post injury. Interestingly, following the crossover in this study, we found that delayed treatment of anatabine showed positive effects by 18 months post-injury in injured mice that did not begin to receive anatabine until 9 months post TBI, specifically reduced Iba1 in corpus callosum. These data demonstrate that late administration of anatabine can be effective, which is encouraging for mTBI patients who typically do not seek medical attention at acute or subacute timepoints after injury.

Overall, the ability of anatabine to suppress astrogliosis/microgliosis, reduce STAT3 and NFκB phosphorylation, mitigate amyloid burden and tau pathology across several neuropathological

models makes it another promising candidate to treat individuals who have been exposed to multiple TBI through their life.

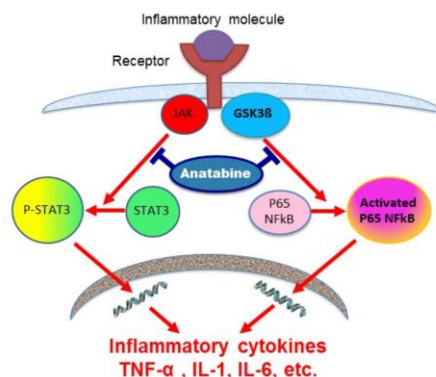


Figure 18. Proposed mechanism of action of anatabine

4.1.2 Rationale of using two models of r-mTBI

One of the proposed reasons of translational failures in drug discovery for TBI is the lack of sufficient interrogation of potential treatments at the preclinical stage (DeWitt et al. 2018; Mohamadpour et al. 2019) including the inadequacy of laboratory approaches to replicate the heterogeneity of the human patient population and the heterogeneity of human brain injuries. In cases of repetitive TBI, additional limitations, such as injury repetitiveness, time interval between the impacts and recovery conditions between the injuries, appear. Therefore, to begin to address these deficiencies we have adopted an overarching mantra that any treatments showing potential in preclinical models of TBI should be explored in multiple different injury paradigms, in order to identify treatments that work in many different scenarios. We therefore decided to test anatabine in two of our well-characterized models of r-mTBI – the 5r-mTBI (previously described in chapters 2 and 3, (Mouzon et al. 2012)) and a chronic repetitive mTBI (cr-mTBI) model in which mice receive 2 hits per week for 3 months (24 hits total) (Ojo et al. 2016). The

two models use the same impact characteristics (depth - 1.0 mm depth, velocity - 5 m/s) but vary in the frequency, length of exposure and inter-concussive interval. In hTau mice, both models trigger neuroinflammation and cognitive deficits (Mouzon et al. 2018; Ojo et al. 2013, 2016).

4.1.3 Rationale for using delayed treatment

More than 80% of individuals with a single mTBI fully recover within days and months; and clinical studies confirm that at 6 and 12 months post mTBI individuals do not differ in cognitive performance from healthy controls (Barker-Collo et al. 2015; Losoi et al. 2016). However, when cognitive deficits persist, especially in cases of repeated concussions, they may lead to memory impairments, mood disorder, anxiety and other problems that manifest years after the impact. Therefore, there is a need for developing delayed treatment that will be effective when administered at later time points after the injury. Unfortunately, this approach is greatly understudied in experimental models of TBI since most studies focus on acute treatment. For example, in a recent review, Bergold summarized treatment studies involving more than 40 anti-inflammatory drugs used in rodents with mild to moderate TBI (Bergold 2016). Almost all studies administered treatment within 5-30 min after TBI with only few extending the window up to 4-6 hours. None of the studies were designed to administer the first dose at times longer than 6 hours. Here, we aim to conduct a more translationally relevant study where r-mTBI mice will be treated with the anti-inflammatory drug anatabine at a delayed timepoint, 3 months after the last injury.

4.1.4 Hypothesis

1) Treatment with anatabine will ameliorate TBI-induced deficits in both models of r-mTBI and, thus, prove to be effective across different models, demonstrating the ability to target heterogeneous nature of TBI and having greater potential to successfully translate to the heterogeneous TBI patient population.

2) Delayed treatment with anatabine will suppress TBI-induced inflammation and related cognitive deficits, again, holding greater translational potential for the human patient population and indicating a wider therapeutic window for TBI than might previously have been anticipated.

4.2 Materials and Methods

4.2.1 Animals

Male and female hTau mice 12-14 weeks old (weight 19-25g) were sourced from Jackson Laboratories (Bar Harbor, ME). The animals were housed under standard laboratory conditions (14-hour light/10-hour dark cycle, $23\pm 1^{\circ}\text{C}$, $50\pm 5\%$ humidity) with free access to food and water. All procedures were carried out under Institutional Animal Care and Use Committee (IACUC) approval and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

4.2.2 Experimental groups and study design

The study was comprised of 2 cohorts of mice that received either 5 r-mTBI (n=34) or chronic-r-mTBI (cr-mTBI, n= 38). In the 5r-mTBI cohort, TBI mice received 5 injuries over 9 days with a 48-hour inter-concussion interval (Mouzon et al. 2012, 2014, 2017, Ojo et al. 2015; Tzekov et al. 2014, 2016). In the cr-mTBI cohort, mice received a total of 24 injuries - 2 injuries each week (with a 3-4 day inter-concussion interval) for the duration of 3 months (Fig.19). Each cohort included four groups: Sham-Vehicle, r-mTBI-Vehicle, Sham-Anatabine, r-mTBI-Anatabine. The distribution of mice between the groups in each cohort is shown in Table 2. Each group included both male and female mice evenly distributed among the groups.

	5r-mTBI	cr-mTBI
Sham-Vehicle	8 (4M,4F)	9 (5M, 4F)
r-mTBI-Vehicle	10 (5M, 5F)	11 (5M, 6F)
Sham-Anatabine	7 (4M, 3F)	8 (5M, 3F)
r-mTBI-Anatabine	9 (6M, 3F)	10 (6M, 4F)

Table 2. Distribution of mice in the groups (M-males, F-females)

For sham/mTBI procedures, all animals underwent anesthesia with 1,5 mL/min of oxygen and 3% isoflurane for 3 min on the heating platform to prevent hypothermia. TBI mice were placed on a heating pad to prevent hypothermia during the injury procedure. The head of each animal was fixed in a stereotaxic frame, and a 5 mm blunt metal impactor was positioned midway to the sagittal suture. The injury was triggered at 5 m/s velocity and 1.0 mm depth, with a dwell time of 200 milliseconds, using a myNeuroLab controller device (Impact One™ Stereotaxic Impactor, Richmond, IL). All TBI mice experienced short-term apnea (<20 sec) and showed no skull fractures. All animals (sham and TBI) were allowed to recover from anesthesia on a heating pad and then returned to their cages with water and soft food access.

Regardless of the paradigm to which mice were assigned, after the last injury/sham procedure, mice were kept under normal housing conditions for 3 months. One week prior to the initiation of treatment, acclimation and baseline levels for Rotarod were recorded. No other manipulations on mice were conducted. After a 3-month period, anatabine was administered to mice of Sham-Anatabine and TBI-Anatabine groups with drinking water in concentration 20mg/kg/day. The volume was calculated based on the average weight of mice prior to treatment (25.5g) and a previously calculated water consumption volume for C57BL/6 mice of 7.4ml/30g (Bachmanov et al. 2002). The solution was changed weekly for 3 months. Placebo mice received regular water. Behavior tests started on day 61 after the initiation of anatabine and included

Rotarod, Barnes Maze, and Elevated Plus Maze as shown in Figure 19. Euthanasia was performed on the 91st day, after the last day of treatment. Behavioral and histological assessments were done by researchers blinded to the experimental groups.

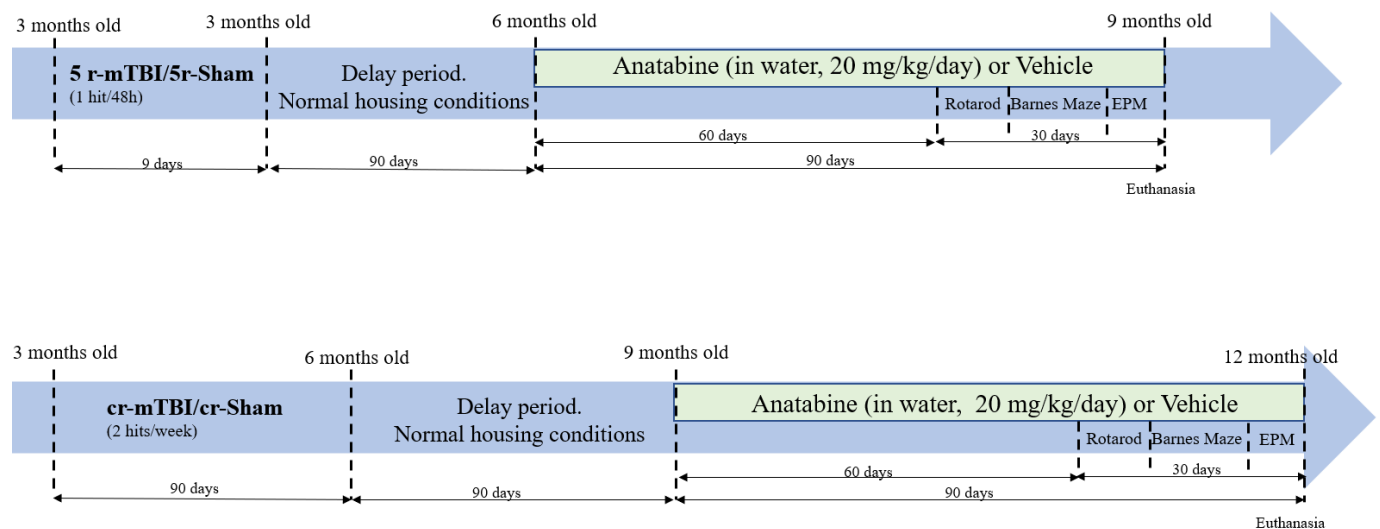


Figure 19. Study design.

Note: The timelines chosen for these two groups were designed in order to match TBI mice by age at first injury (3 months old). This resulted in the mismatch of animals' age at euthanasia (5r-mTBI - 9 months; cr-mTBI – 12 months).

4.2.3 Behavior

Behavior was assessed during the last month of treatment and included Rotarod, Barnes Maze (BM) and Elevated Plus maze (EPM). A detailed procedure for each testing is described in the Methods section of the second Chapter. Briefly, Rotarod acclimation and baseline levels

were recorded during a delay period before the initiation of treatment. Acclimation included 3 trials for 5 min without acceleration while baseline trial was performed using acceleration mode 5 to 50 rpm. Rotarod assessment started on day 61 after the beginning of Anatabine treatment and was performed every other day during a 7-day trial. Each animal underwent 3 trials for 3 min with 5 min interval rest. After the final day of rotarod testing, cognitive function was evaluated using the BM. For a period of 6 days, four trials were given per day, with mice starting from one of four cardinal points on each trial. On the 7th day, a single probe trial lasting 60 sec was performed with the mouse starting from the center of the maze and the target box removed. Disinhibition behavior was assessed using the *EPM* day prior to euthanasia. Mice were placed in the middle of the plus-shaped maze elevated 80 cm above the ground with 2 open and 2 closed arms perpendicular to each other in a brightly lit room. Animal movement was recorded during a 5 min trial. Each animal underwent only one trial. The center point of the mouse determined by Ethovision XT14 tracking was used to decide the current arm of maze the mouse occupied. The time spent in open vs closed arms was calculated.

4.2.4 Tissue processing and immunofluorescence

At 6 months post last mTBI/sham injury, mice were anesthetized with isoflurane and perfused transcardially with phosphate-buffered saline (PBS) with heparinized PBS, pH-7.4. Brains were removed and post fixed in a solution of 4% paraformaldehyde at 4°C for 48 h, dehydrated in graded ethanol solutions, cleared in histoclear, and embedded in paraffin. Serial sections (6 µm thick) were cut onto positively charged glass slides and boiled in citrate buffer (10mM pH 6.0) for antigen retrieval, followed by incubation with the following antibodies: *GFAP/NFκB/RZ3*. Fluorescent staining was performed with the antibodies for astroglial marker

GFAP (7857983, Aves Labs), NFkB (3034, Cell Signaling) and phosphorylated tau at Thr231 (RZ3). Following rehydration, antigen retrieval was performed by heating slides in citric acid buffer for 7 min in a microwave oven. Next, slides were washed with PBS and transferred to a Sudan Black solution for 15 minutes to prevent autofluorescence. Slides were then blocked for 1 h with 10% donkey serum solution in PBS, and primary antibodies for GFAP (1:500), NFkB (1:500) and RZ3 (1:500) were applied overnight. On the next day, secondary antibodies AlexaFluor488 (A21202, Life Technologies) and AlexaFluor647 (ab175477, Abcam) were applied. Slides were mounted with ProLong Gold Antifade 4',6-diamidino-2-phenylindole (DAPI) Mount. Imaging was performed using a confocal microscope (LSM 800 Zeiss) at 20x magnification. Quantification of the fluorescent images was performed using LSM 800 Zeiss and the intensity of fluorescence was measured for each antibody. The same area of interest was applied to each image and the background signal was subtracted from the obtained values.

4.2.5 Statistical analysis

All experimental data were analyzed using JMP 12 and PRISM Software. The data were checked for normality using Skewness-Kurtosis and Goodness of Fit. If normal, parametric methods (t-test and one-way ANOVA) were applied to calculate the significance between different experimental groups ($p < 0.05$ were considered significant). The Shapiro-Wilk test was used if data were not normally distributed. All data were transformed to logarithm or square root, when required, to reach normality before further analysis. Repeated-measure analysis of variance (MANOVA) was used to analyze continuous performance of mice in the Barnes Maze and Rotarod ($p < 0.05$ is significant). Error bars represent the standard error of the mean.

4.3 Results

4.3.1 Motor assessment

Rotarod assessment showed a 37.5% TBI-induced decrease in sensorimotor performance of cr-mTBI-vehicle compared to cr-sham-vehicle group (Fig.20). No TBI effect was observed in 5r-mTBI-vehicle vs 5r-sham-vehicle mice (Fig.20 B). The cr-mTBI-vehicle mice had a significantly lower latency to fall compared to their controls during a 7-day trial (Fig.20 A; cr-mTBI-vehicle vs cr-sham-vehicle: 128 ± 3 sec vs 206 ± 17 sec; $p < 0.0001$, MANOVA). In the 5r-mTBI mice, an overall motor performance was lower in the 5r-mTBI-vehicle mice vs 5r-sham-vehicle, however, when normalized to each group baseline, this significance disappeared ($p > 0.05$, MANOVA). Treatment with anatabine in the cr-mTBI mice increased latency to fall compared to vehicle treated cr-mTBI mice (cr-mTBI-anatabine vs cr-mTBI-vehicle: 206 ± 23 sec vs 128 ± 3 sec; $p < 0.0001$, MANOVA). In the 5r-mTBI animals, no anatabine-dependent response was observed in the TBI mice.

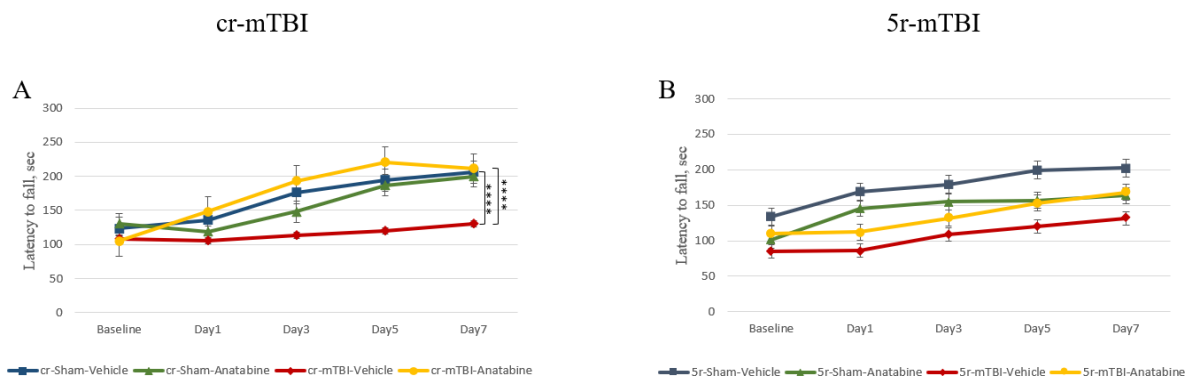


Figure 20. Evaluation of the effect of anatabine on motor performance Anatabine treatment improved motor function outcomes in the cr-mTBI (A) but not 5r-mTBI (B). Mice in the cr-mTBI-vehicle group showed a significant decrease in their performance compared to the cr-sham-vehicle (A; $p < 0.0001$). This effect was not observed in the 5r-mTBI-vehicle mice (B). Treatment with anatabine ameliorated motor deficits in the cr-mTBI mice (cr-mTBI-anatabine vs

cr-mTBI-vehicle, $p<0.0001$). (B) In 5r-mTBI mice treated with anatabine, no differences were observed in the latencies compared to the 5r-mTBI-vehicle ($p>0.05$). All data were analyzed using MANOVA.

4.3.2 Memory assessment

cr-mTBI: The acquisition trial showed an overall decrease in cumulative distance, distance traveled and latency to enter the box in all groups. cr-mTBI-vehicle mice showed a 68% increase in the cumulative distance compared to the cr-sham-vehicle by the end of a 6-day acquisition trial (Fig.21 A; $p<0.05$, MANOVA). For distance traveled, cr-mTBI-vehicle mice showed a 33% increase compared to cr-sham-vehicle mice (Fig.21 B; $p<0.05$, MANOVA). In the cr-mTBI-vehicle mice, latency to enter the box was 43% increased compared to cr-sham-vehicle (Fig. 21 C; day 6 $p<0.01$, one-way ANOVA). Latency was not different between cr-sham-vehicle and cr-mTBI-vehicle until day 4 (day 4 $p<0.001$, day 5 $p<0.001$, day 6 $p<0.01$, one-way ANOVA). cr-mTBI-anatabine mice had a 65% decrease in cumulative distance compared to cr-mTBI-vehicle group ($p<0.05$, MANOVA). For distance traveled, cr-mTBI-anatabine mice exhibited a 30% decrease compared to cr-mTBI-vehicle. Treatment with anatabine significantly reduced latency to enter the box in the injured mice starting on day 2 of the acquisition trial (cr-mTBI-anatabine vs cr-mTBI-vehicle: day 2 $p<0.01$, day 3 $p<0.01$, day 4 $p<0.001$, day 5 $p<0.01$, day 6 $p<0.01$, one-way ANOVA). During the probe trial, cr-mTBI-vehicle mice spent 90% longer time to locate the target hole compared to the cr-sham-vehicle (Fig. 22 A; cr-mTBI-vehicle vs sham-vehicle: $28.3\pm 21\text{sec}$ vs $2.6\pm 1.2\text{sec}$; $p<0.05$, one-way ANOVA). In the cr-mTBI-anatabine mice, mean time was 82.3% decreased compared to cr-mTBI-vehicle (cr-mTBI-anatabine vs cr-mTBI-vehicle: $4.9\pm 4.2\text{sec}$ vs $28.3\pm 21\text{sec}$; $p<0.05$, one-way ANOVA).

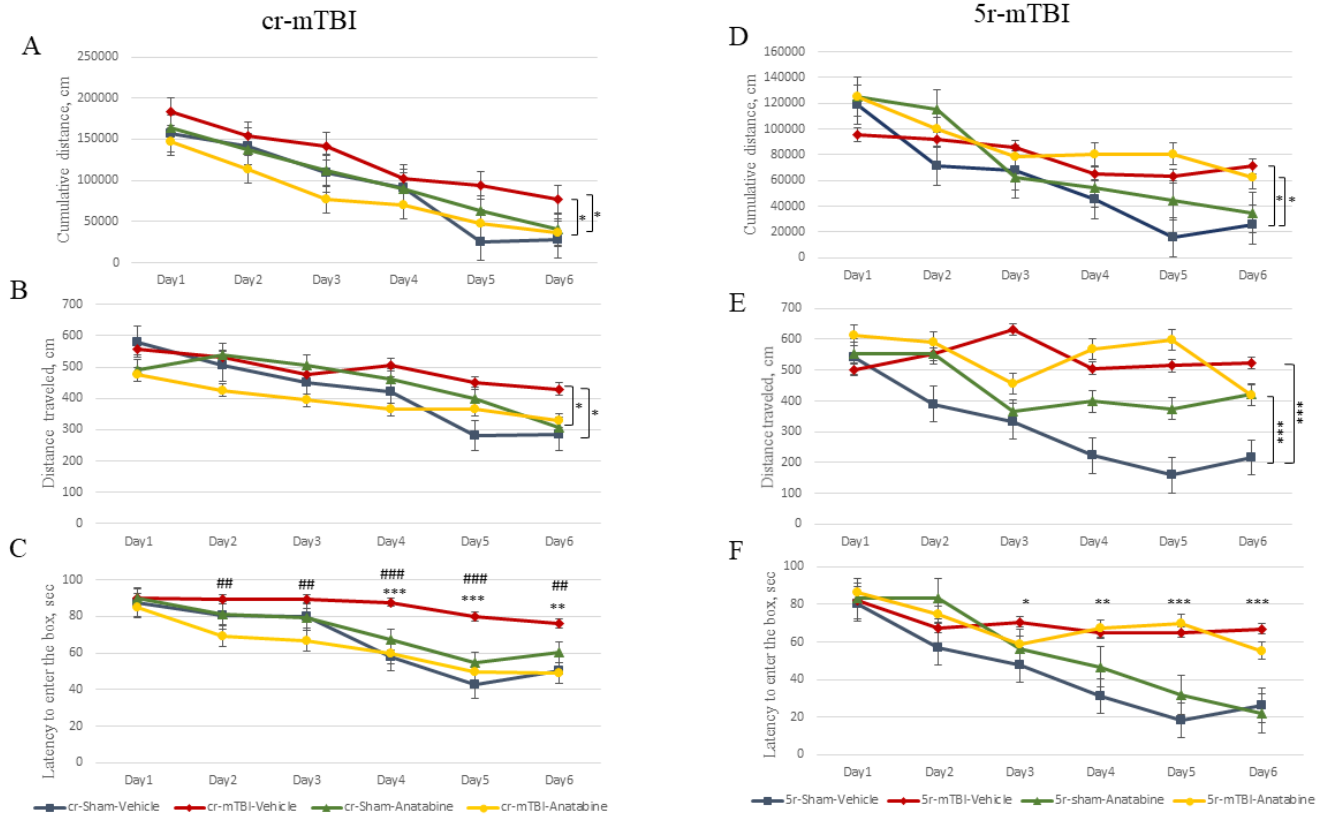


Figure 21. Evaluation of learning and memory in cr-mTBI and 5r-mTBI mice. Anatabine treatment improved spatial memory deficits in the cr-mTBI but not 5r-mTBI mice. In the cr-mTBI cohort, cr-mTBI-vehicle mice exhibited longer cumulative distance (A; $p < 0.05$), distance traveled (B; $p < 0.05$), and latency to enter the box on days 4-6 (C) compared to cr-sham vehicle. Similarly, in the 5r-mTBI cohort, 5r-mTBI-vehicle mice exhibited longer cumulative distance (D; $p < 0.05$), distance traveled (E; $p < 0.001$), and latency to enter the box on days 3-6 (F) compared to 5r-sham vehicle. cr-mTBI-anatabine mice showed a decrease in cumulative distance (A; $p < 0.05$), distance traveled (B; $p < 0.05$) and latency (C) compared to cr-mTBI-vehicle mice. Anatabine did not attenuate memory deficits in 5r-TBI-anatabine group. Statistical significance for the cumulative distance and distance traveled were analyzed using MANOVA; the latency for each day were analyzed using one-way ANOVA.

5r-mTBI: The acquisition trial showed an overall decrease in the cumulative distance, distance traveled, and latency to enter the target box for all experimental groups over a 6 day

period. 5r-mTBI-vehicle mice showed a 67% increase in the cumulative distance compared to the 5r-sham-vehicle by the end of a 6-day acquisition trial (Fig. 21 D; $p<0.05$, MANOVA). Similarly, in the 5r-mTBI-vehicle mice, distance traveled was 52% increased (Fig. 21 E; $p<0.001$, MANOVA) and latency to enter the box was 57.8% increased, compared to 5r-sham-vehicle (Fig. 21 F; day 6 $p<0.001$, one-way ANOVA). Latency to enter the box was increased in the 5r-mTBI-vehicle mice compared to 5r-sham-vehicle starting day 3 after the initiation of the trial (days 3 $p<0.05$, day 4 $p<0.01$, day 5-6 $p<0.001$, one-way ANOVA). No anatabine-induced improvements were shown for any of these parameters in the 5r-mTBI group (5r-mTBI-anatabine vs 5r-mTBI-vehicle: cumulative distance $p>0.05$, distance traveled $p>0.05$, latency $p>0.05$). In the probe trial, latency to locate the target hole was increased in the 5r-mTBI-vehicle mice vs 5r-sham-vehicle (Fig. 22 B; $p<0.05$, one-way ANOVA) but no anatabine-induced decrease in the 5r-TBI mice was shown compared to 5r-mTBI-vehicle ($p>0.05$, one-way ANOVA).

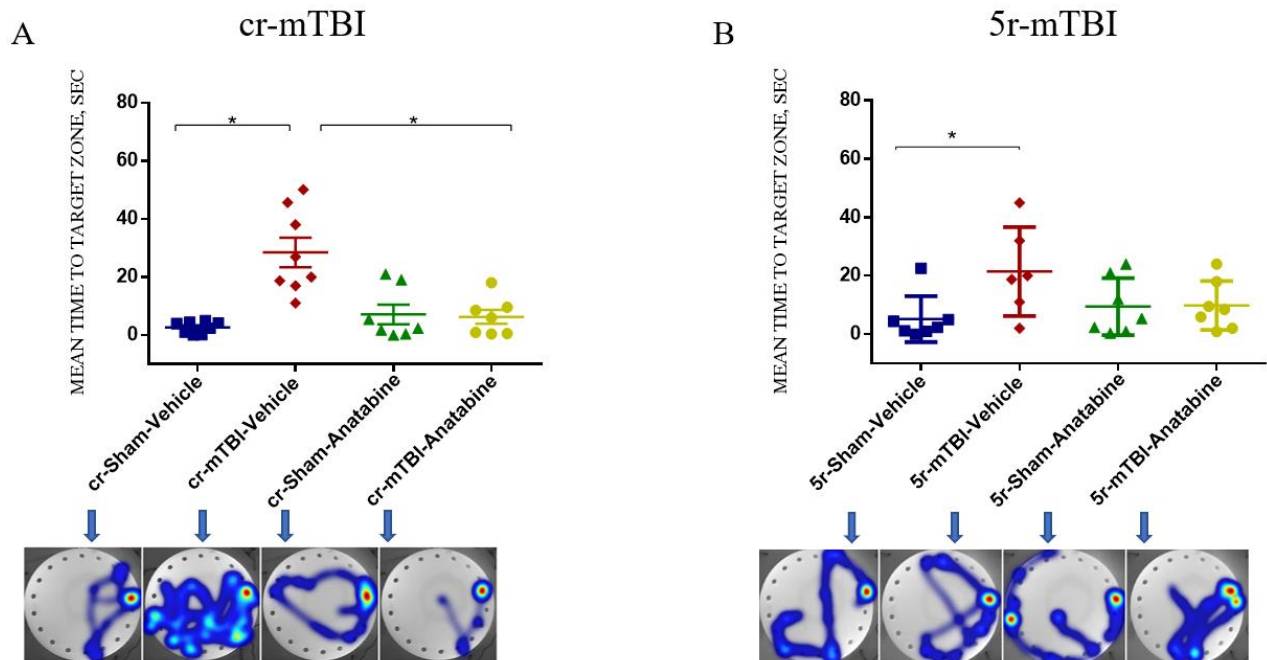


Figure 22. Probe trial in the cr-mTBI and 5r-mTBI mice. Mean time to enter the target zone was decreased in the cr-mTBI but not 5r-mTBI after anatabine treatment. A – In the cr-mTBI group, TBI induced a significant increase in mean time compared to sham-vehicle ($p<0.05$), which was decreased in the cr-mTBI-anatabine mice vs cr-mTBI-vehicle ($p<0.05$). B – In the 5r-mTBI group, 5r-mTBI-vehicle mice had an increase in the mean time compared to 5r-sham-vehicle ($p<0.05$) but no anatabine induced decrease was detected in the 5r-mTBI-anatabine mice. Statistical significance was analyzed using one-way ANOVA.

4.3.3 Disinhibition assessment

EPM was used in both models of r-mTBI to measure disinhibition, which reflects a spectrum of human behavior including aggression, impulsivity, poor social interaction and risk assessment skills, all or some of which are often reported post injury. EPM analysis showed a significant TBI effect in the 5r-mTBI but not cr-mTBI mice. In the 5r-mTBI group, 5 r-mTBI-vehicle mice spent significantly more time in the open arm compared to the 5r-sham-vehicle indicating disinhibition behavior (Fig. 23; $p<0.05$, one-way ANOVA). Treatment with Anatabine significantly reduced time spent in open arms in the 5r-mTBI mice ($p<0.01$, one-way ANOVA). In the cr-mTBI mice, no differences were observed between the groups.

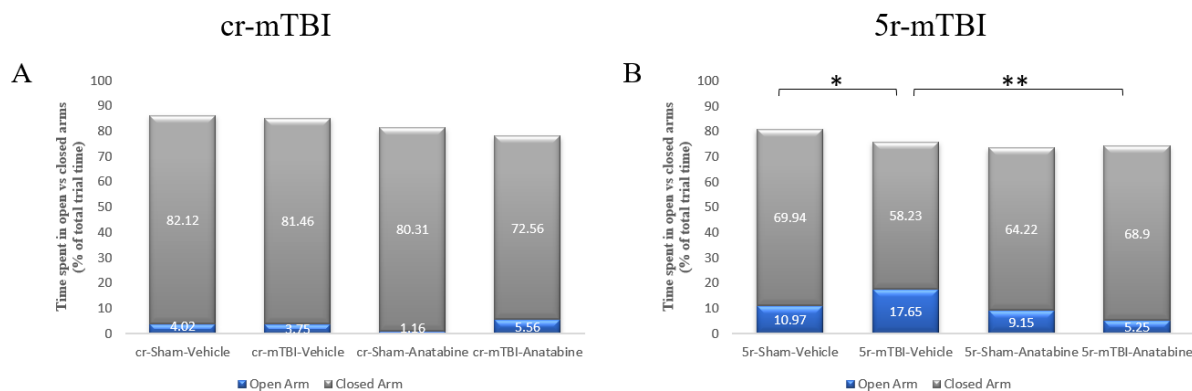


Figure 23. Evaluation of disinhibition in the cr-mTBI and 5r-mTBI mice. Disinhibition was suppressed by anatabine in the 5r-mTBI but not cr-mTBI mice. A – in the cr-mTBI cohort, TBI did not produce disinhibition behavior compared to sham mice. There was no difference between cr-mTBI-vehicle and cr-mTBI-anatabine groups. B – in the 5r-mTBI cohort, TBI increased time spent in the open arm compared to sham-vehicle ($p < 0.05$). Treatment with anatabine increased time spent in the closed arm in the 5r-mTBI mice compared to 5r-mTBI-vehicle ($p < 0.01$). Statistical significance was analyzed using one-way ANOVA.

4.3.4 Immunohistochemistry

In the cr-mTBI but not 5r-mTBI mice, Iba1 signal was increased in the corpus callosum in the injured mice compared to their respective controls (Fig. 24 ; 5r-mTBI-vehicle vs 5r-sham-vehicle $p > 0.05$, cr-mTBI-vehicle vs cr-sham-vehicle $p < 0.05$, one-way ANOVA). In the cr-mTBI mice, anatabine resolved microglia activation close to cr-sham-vehicle levels (Fig. 24 B,C; cr-mTBI-anatabine vs cr-mTBI-vehicle $p < 0.05$, cr-mTBI-anatabine vs cr-sham-vehicle $p > 0.05$, one-way ANOVA).

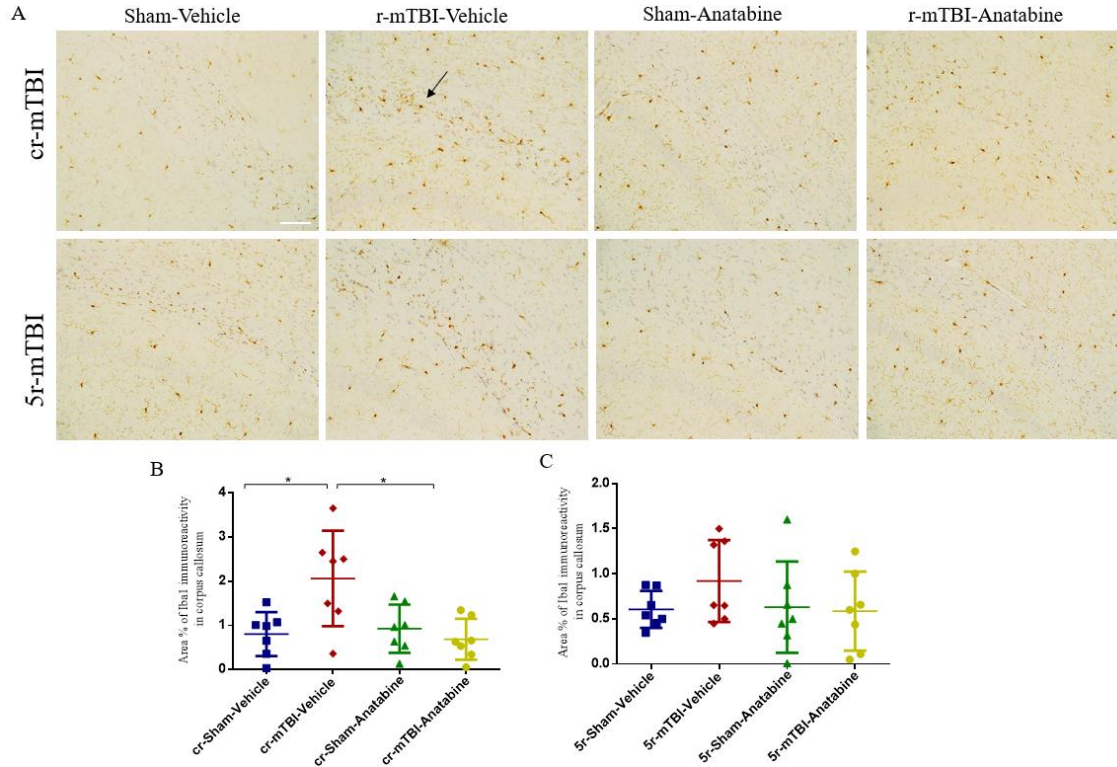
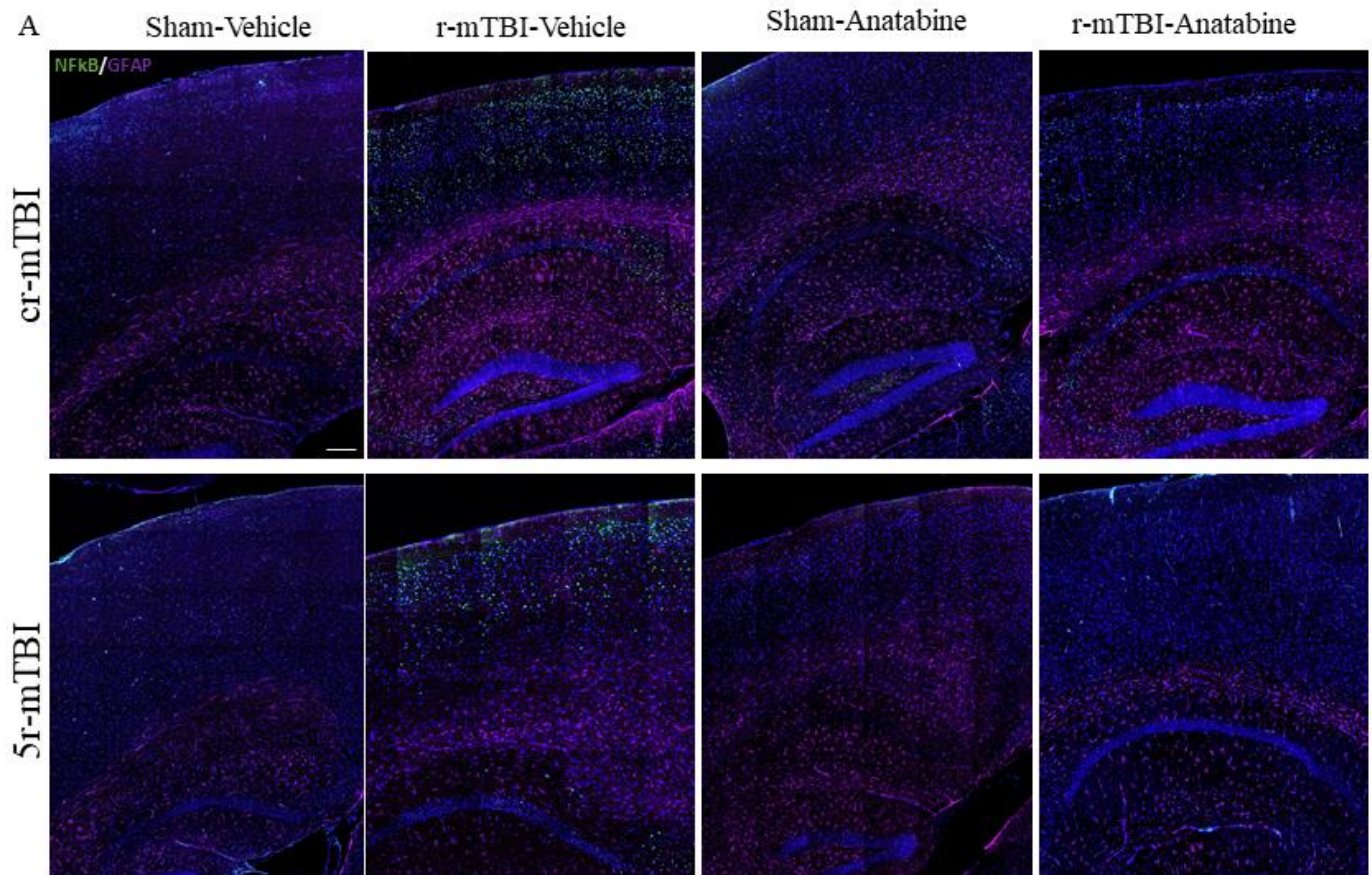


Figure 24. Evaluation of the effect of anatabine on microglia. A – representative images of Iba1 in corpus callosum in the 5r-mTBI and cr-mTBI mice. B – cr-mTBI-vehicle mice had an increase in Iba1 signal compared to cr-sham-vehicle ($p < 0.05$). C – in 5r-mTBI-vehicle mice, no signs of increased Iba1 were found. Anatabine decreased Iba1 in cr-mTBI mice (cr-mTBI-anatabine vs cr-mTBI-vehicle $p < 0.05$) but not in 5r-mTBI. Data analyzed using one-way ANOVA. Scale bars equal 20 μ m.

Immunofluorescent assessment for the astroglial marker GFAP revealed an increase in astrogliosis in the injured mice compared to the sham (Fig. 25 A). In both 5r-mTBI and cr-mTBI groups, a strong glial response was detected in the hippocampus and corpus callosum but not in the cortex. Quantitative analysis showed a significant increase in the area % of GFAP cells in the corpus callosum of the r-mTBI mice compared to sham-vehicle mice (Fig. 25 D: 5r-mTBI-vehicle vs 5r-sham-vehicle $p < 0.001$; Fig. 25 B: cr-mTBI-vehicle vs cr-sham-vehicle $p < 0.001$).

After treatment with anatabine, both 5r-mTBI and cr-mTBI showed a significant reduction in GFAP immunoreactivity in corpus callosum (5r-mTBI-anatabine vs 5r-mTBI-vehicle $p<0.01$; cr-mTBI-anatabine vs cr-mTBI-vehicle $p<0.01$, one-way ANOVA). Immunoreactivity for NFkB was detected in the cortex and hippocampal areas CA1, CA3, and DG. In the cortex, quantitative analysis for NFkB revealed an increase in both cohorts in the r-mTBI groups compared to their respective controls: 5r-mTBI (Fig. 25 E: 5r-mTBI-vehicle vs 5r-sham-vehicle $p<0.0001$, one-way ANOVA) and cr-mTBI (Fig. 25 C: cr-mTBI-vehicle vs cr-sham-vehicle $p<0.0001$, one-way ANOVA). Images at higher magnification (20X) revealed a NFkB/DAPI colocalization in the r-mTBI mice in the CA1 area of hippocampus and throughout cortex (Fig. 26). Their increase was attenuated after treatment with anatabine in all areas (quantitative analysis for cortex: 5r-mTBI-anatabine vs 5r-mTBI-vehicle $p<0.0001$; cr-mTBI-anatabine vs cr-mTBI-vehicle $p<0.001$).



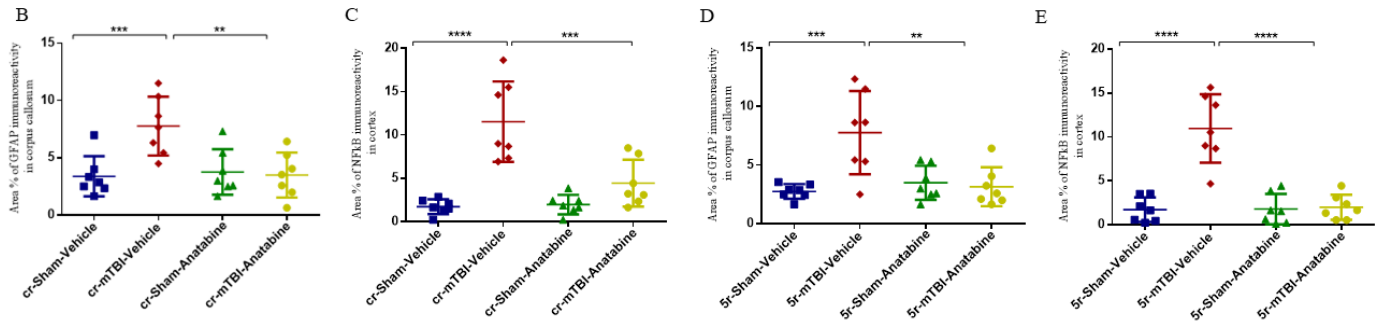


Figure 25. Immunofluorescent analysis of **GFAP** and **NFkB**. A – representative images of **NFkB**/**GFAP** in cortex, corpus callosum and hippocampus. In the cr-mTBI group, TBI produced a significant increase of **GFAP** in corpus callosum (B, $p < 0.001$) and of **NFkB** in cortex (C, $p < 0.0001$) compared to cr-sham-vehicle. Similarly, in the 5r-mTBI group, TBI induced **GFAP** (D, $p < 0.001$) and **NFkB** (E, $p < 0.0001$) immunoreactivity compared to 5r-sham-vehicle mice. Treatment with anatabine decreased **GFAP** (cr-mTBI $p < 0.01$; 5r-mTBI $p < 0.01$) and **NFkB** (cr-mTBI $p < 0.001$; 5r-mTBI $p < 0.0001$) in r-mTBI mice compared to the respective r-mTBI-vehicle mice. Data analyzed using one-way ANOVA. Scale bar equal 200 μm .

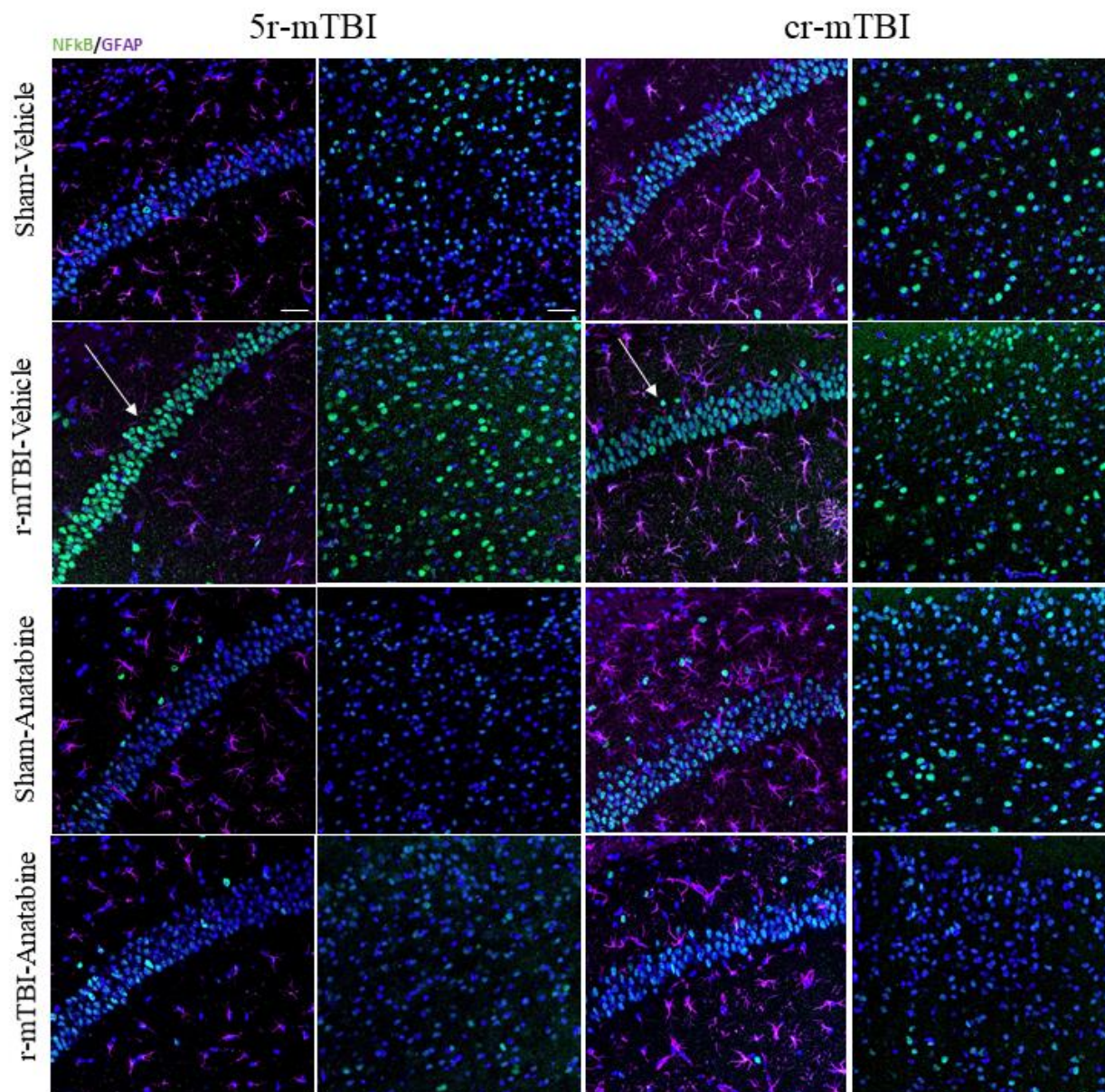


Figure 26. Representative images of **NFkB** in cortex and CA1 area of hippocampus in 5r-mTBI and cr-mTBI mice. Arrows indicate nuclear localization of **NFkB**. Scale bars equal 20 μ m.

Immunofluorescent analysis for phosphorylated tau showed a significant increase of RZ3 signal in cortex (Fig.27 A) of both 5r-mTBI and cr-mTBI mice compared to their respective sham-vehicle mice (Fig. 27 B: 5r-mTBI-vehicle vs 5r-sham-vehicle: $p < 0.05$; Fig. 27 C: cr-

mTBI-vehicle vs cr-sham-vehicle $p < 0.001$, one-way ANOVA). Anatabine treatment decreased RZ3 fluorescent intensity in the 5r-mTBI (vs 5r-mTBI-vehicle $p < 0.05$, one-way ANOVA) and cr-mTBI (vs cr-mTBI-vehicle $p < 0.001$, one-way ANOVA).

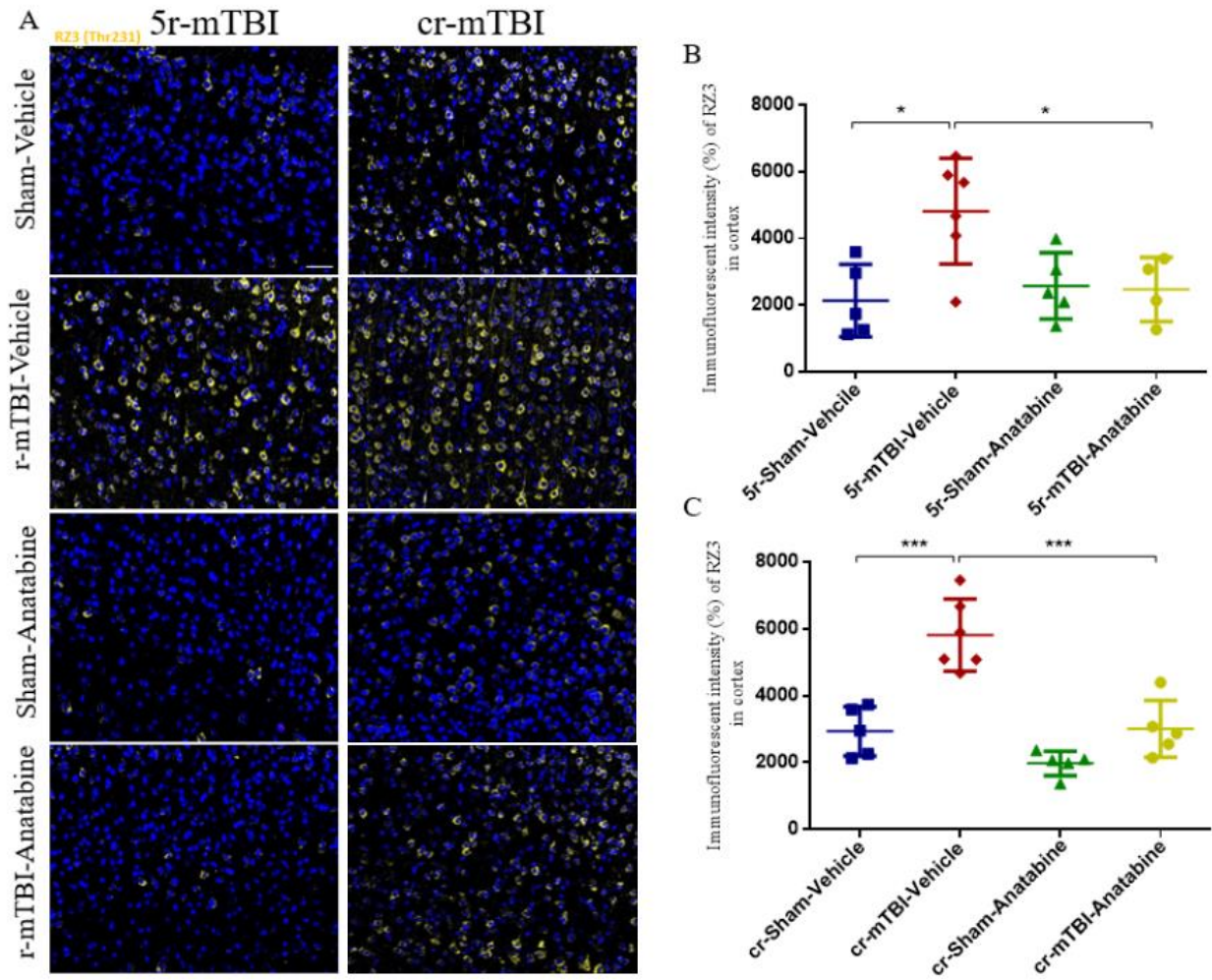


Figure 27. Evaluation of the effect of anatabine on RZ3 in cortex. A – representative images of RZ3 in cortex. TBI induced an increase of RZ3 in 5r-mTBI (B, $p < 0.05$) and cr-mTBI (C, $p < 0.001$) mice compared to the respective sham-vehicle. In the r-mTBI-anatabine mice, RZ3 was decreased in both 5r-mTBI ($p < 0.05$) and cr-mTBI ($p < 0.001$) mice compared to the respective r-mTBI-vehicle. Data analyzed using one-way ANOVA. Scale bars equal 20 μ m.

4.4 Discussion

This chapter focused on the delayed treatment with anatabine in hTau mice in two different models of r-mTBI. We found that anatabine decreased GFAP, Iba1, NFkB and p-tau in both models, however its effect on behavior was mostly demonstrated in the cr-mTBI model, but not in the 5r-mTBI mice.

Both 5-rmTBI and cr-mTBI mice (vehicle treated) exhibited strong injury-dependent astrogliosis in the corpus callosum and hippocampus 6-months post last injury/sham. However, no reactive astrocytes were observed in cortex within the injury site, a pathology commonly found at sub-acute phase in our models (Mouzon et al., 2012). Such temporal alterations probably indicate that accumulation of astrocytes in the cortex shortly after TBI is associated with mechanical deformation of tissue, and it quickly resolves after the termination of mechanical stress. On the contrary, white matter has been shown to be more vulnerable to TBI leading to long-lasting pathology (Braun et al. 2017). White matter is comprised of interconnected axon tracts that are especially susceptible to stretch forces at the grey-white matter junctions. In fact, most axonal injury in white matter is caused not by shear and stress forces but as a result of white matter atrophy, demyelination and other secondary injury events (Büki and Povlishock 2006). These processes have been shown to sustain for over one year in different rodent models of TBI (Bramlett and Dietrich 2002) and TBI patients (Johnson et al. 2013), and to be linked to cognitive deficits (Alhilali et al. 2015; Bramlett and Dietrich 2015; Kinnunen et al. 2011). We also observed an increased phosphorylation of NFkB in both our models at 6 months post-injury. NFkB is a transcription factor that is involved in the regulation of immune response and inflammation. Under baseline conditions, NFkB is found in cytosol but upon activation, it translocates into nucleus where it binds specific DNA sequences and promotes

the expression of inflammatory cytokines (Nennig and Schank 2017). Our data confirm a TBI-induced nuclear localization of NFkB in both groups – 5r-mTBI and cr-mTBI. As we expected, tau phosphorylation was also upregulated in the cortex and the CA1 area of the hippocampus at 6 months post injury. We have previously shown an increase in total and p-tau (Thr 231) in cr-mTBI hTau mice at 3 months post-injury (Ojo et al. 2016) but these data represent our first time reporting a similarly persistent p-tau signal in the 5r-mTBI hTau mice. Our data suggest that 5 mild hits are sufficient to increase phosphorylation of tau to a degree shown in the model with 24 hits.

Treatment with anatabine for 3 months after a delay of 3 months post-last injury attenuated TBI-induced pathology in both models of r-mTBI. We observed a decrease of GFAP, Iba1, NFkB and p-tau, markers that were increased in response to TBI but not in sham mice. These data are supported by our previous findings where at 9 months post 5r-mTBI, anatabine reduced inflammation and improved cognitive functions in wild-type mice (S. Ferguson et al. 2016). Interestingly, in our previous study, we observed that following an initial 9 months of treatment, a 9-month cessation of anatabine resulted in a return of the injury-dependent Iba1 signal indicating that microgliosis was suppressed but not completely removed by anatabine. Thus, anatabine does not target the underlying causes of inflammation but exhibits a modulating effect for the period of treatment. However, a 9-month delayed treatment with anatabine successfully reduced Iba1 to sham levels at the 18-month time points, which gave incentive for our study described in this chapter.

At 6 months post TBI, both models exhibited injury-induced memory and learning deficits whereas, unexpectedly, only the 5r-mTBI, but not the cr-mTBI mice, showed disinhibition behavior. A long-lasting disinhibition (time spent in the open arm) was found in

other models of 5-hit mTBI in WT mice (Gold et al. 2018) but no study has addressed the effect of >20 hits on such behavior. The lack of disinhibitory behavior in the cr-mTBI, a more severe model than 5r-mTBI, requires further investigation. The effect of anatabine on motor and cognitive outcomes showed inconsistent results between the two models. In the cr-mTBI mice, anatabine mitigated both sensorimotor decline and memory loss, whereas in the 5r-mTBI group anatabine did not exhibit therapeutic effect on the same parameters. However, in 5r-mTBI but not cr-mTBI mice, anatabine decreased disinhibition, the only TBI-induced behavior changes that were detected in this model. So far, we are unable to elaborate on the diverse effect of anatabine on cognitive improvements, especially when ameliorated pathology was shown in both models. A possible explanation may be that anatabine acts through the alternative mechanisms which differ between the models due to the final age of mice and the injury model. Anatabine is an agonist of $\alpha 7$ subunit of nAChR which is involved in the regulation of calcium signaling (Shen and Yakel 2009). Calcium dysregulation has been shown a key feature of secondary injury involved in cellular apoptosis and oxidative stress. Further investigation of its response to anatabine in TBI may provide explanation for the dual effect of the drug seen in our models.

The largest platform so far that was designed to compare the efficacy of potential treatments across different injury models in order to enhance the translation of therapies from rodents to men is Operation Brain Trauma Therapy (OBTT) consortium (Kochanek et al. 2018). Through the extensive literature review, they identified 10 therapies that were further tested across rat models of FPI, CCI and penetrating ballistic-like brain injury (PBBi). To assess therapeutic efficacy, they developed a scoring matrix (max. 22 points) based on the primary outcome metrics including sensorimotor, cognition, neuropathology and biomarkers (GFAP and UCHL-1). To date, the results of the first 5 treatments – nicotinamide, erythropoietin,

cyclosporine, simvastatin, and levetiracetam – showed that 4 of them underperformed relative to initial studies in individual models of TBI and only levetiracetam reached 10 (out of 22) points across different models (Kochanek et al. 2016). The follow-up testing of levetiracetam and the remaining 5 treatments (glibenclamide, minocycline, AER-271, amantadine, VA64) are ongoing. Overall, this study demonstrated that the most promising preclinical therapeutic candidates fail to reproduce similar results across various models. Despite these findings, OBTT approach has several limitations that may undermine its translational potential. First, the TBI models used represent very different mechanisms ranging from diffuse (FPI) to focal (CCI, PBBI), each of them causing distinct pathology meaning that successful treatment for one type receives a score which does not properly reflect its translational potential within that category of injury. Second, all treatments were administered at the very acute time points ranging from 15 minutes to 24 hours post-TBI. This time window may have missed treatments that require chronic administration relevant for cases of human mild and sometimes even moderate TBI.

We would like to acknowledge that our study described in this chapter also has several limitations and the comparison between two models of r-mTBI should be performed carefully. First, the age of mice at the time of treatment and euthanasia was different between the cohorts. Due to the longer duration of cr-mTBI paradigm (12 weeks) than 5r-mTBI (9 days), mice that entered the study at 3 months of age, were older in the cr-mTBI group (12 months) than 5r-mTBI (9 months) by the end of the study (Table 3). The age difference at each step (i.e. end of injury, beginning of treatment, behavior assessment, euthanasia and tissue collection) might have contributed to the variations of treatment outcomes.

Age	5r-mTBI	cr-mTBI
<i>at first injury</i>	3 months/12 weeks	3 months/12 weeks
<i>at last injury</i>	>3 months/13 weeks	6 months/24 weeks
<i>at first dose of anatabine</i>	6 months/ 25 weeks	9 months/36 weeks
<i>at behavior assessment</i>	8 months/33 weeks	11 months/44 weeks
<i>at last dose of anatabine</i>	9 months/41 weeks	12 months/52 weeks
<i>at euthanasia</i>	9 months/41 weeks	12 months/52 weeks

Table 3. Age of mice across the major intervention steps

Second, our study was limited to a single treatment time-point and, thus, does not provide a clear picture of the time-dependent treatment effect. Administration of additional time points of treatment with anatabine post-injury may provide a better picture of its effect on chronic outcomes.

Overall, our study provides evidence that certain drugs can vary therapeutically between different models of TBI, and it may account for the failures in clinical translation. To better investigate heterogeneity of different models and treatment paradigms, we plan to conduct a proteomic analysis of mice across different studies described in Chapter 3 and Chapter 4. Identification of TBI- and treatment-related variations on molecular level is an important undertaking.

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Chapter 5 - Proteomic approach to the identification of phosphorylated proteins across different models of r-mTBI and treatment paradigms in order to determine common therapeutic targets.

5.1 Introduction

Proteomics is a technique that allows the identification of a large number of proteins, across a wide dynamic range, in tissue extracts or biofluids. Mass spectrometry (MS)-based proteome analysis of TBI brain tissue may identify novel biomarkers, pathways, protein-protein interactions and potential targets associated with brain injury (Denslow et al. 2003). MS proteomics requires no prior knowledge of the proteins being identified and thus provides an unbiased approach to finding TBI-associated changes. So far, only a few preclinical studies have conducted proteomic analysis in models of TBI, and even fewer after mild TBI; moreover, to the best of our knowledge, none has assessed TBI proteomics in response to treatment (Sowers et al. 2018; Chen et al. 2018; Song et al. 2018). These studies reported that mechanisms dysregulated acutely after a single mTBI (3-24 hours) included microtubule organization, axon guidance and myelination, synaptic functions and calcium signaling (Chen et al. 2018; Song et al. 2018). Meanwhile, at 30 days and 15 weeks, pathways involving neurogenesis and neuronal development and differentiation were altered (Chen et al. 2018). Moreover, we have previously shown 13 different proteins that were changing over multiple time points post injury (24 hour, 3, 6, 9, 12 months) in the 5r-mTBI WT mice (Joseph O. Ojo et al. 2018). These proteins included serine protease and metalloproteinase enzymes, immunoglobulins, and inflammatory proteins. The associated canonical pathways modulated after r-mTBI in our model involved complement system, production of nitric oxide and oxygen species, and LXR/LXR pathway, which is linked

to inflammation, oxidative stress and lipid biogenesis. The direction of changes involved an acute trend (24 hours) in the TBI vs sham mice toward a decrease at 3 month and a gradual increase at 6, 9, and 12 months post-injury, indicating non-linear progression of pathology. Significant differences have also been shown between single and r-mTBI, where mechanisms unchanged after single mTBI, such as cell adhesion or cAMP signaling pathways, were significantly *downregulated* after r-mTBI (Song et al. 2018). Another factor that may contribute to proteomic variations in the models of TBI is age at the time of injury. However, one study that measured the effect of moderate TBI on proteomic profile in old (21 months) or young/juvenile (5 weeks/16 weeks) rats showed limited age-induced variations (Mehan and Strauss 2012). Mehan and Strauss identified a total 19 proteins (e.g. heat shock proteins (HSP), ApoE, vimentin, etc) that were equally *upregulated* after TBI across all age groups relative to the age-matched controls with only one protein - serum albumin protein (SAP) – that appeared to be increased only after TBI in the young but not old rats due to higher baseline levels of this protein in the aged sham rats. These data don't support strong proteomic variations in the age-dependent TBI reponse, however their study was limited to a very small number (19) of the analyzed proteins and might not be representative of real proteome changes. Overall, there are limited data on TBI proteomics, and inter-laboratory variations in the MS-based protocols, animal strains, euthanasia procedures and other factors confounds any collective interpretation. Nonetheless, such studies clearly have the potential to provide important information about protein changes across different ages, injury models and time points, which could reveal therapeutic targets and biomarkers for diagnosis and theragnosis.

In this chapter, we present our first attempt our proteomic profiling from the two different injury paradigms and two treatments described in Chapters 3 and 4. In addition to

investigating the response to injury, these studies provided us with the opportunity to identify the influence of treatment on the response to injury, in settings where therapeutic benefits were demonstrated. As previously discussed, for effective clinical translation from preclinical studies investigation of different models is needed, and identification of molecular level responses that correlate with beneficial treatments in different models may help us to hone the therapeutic targets. Given limited time, we began with a phosphoproteomic approach, which will be complemented by a total proteomics analysis in future studies. Given the possible confounder of age at injury, at this time we focused on our studies of young mice receiving injury. Samples were included from the following studies: 5r-mTBI/Nilvadipine (chapter 3), 5r-mTBI/Anatabine, and cr-mTBI/Anatabine (chapter 4). These studies varied by several parameters: 1) injury model (5r-mTBI or cr-mTBI); 2) drug (nilvadipine or anatabine); 3) time at the beginning of treatment (acute vs chronic/delayed); 4) age of mice at the time of treatment and euthanasia. Previously, we showed that such factors as age, injury model and treatment paradigm contribute to variations in the pathological outcomes and neurorecovery. Identification of any common phosphoproteomic responses, despite all these variables, could reveal possible therapeutic targets that can be efficient across TBI heterogeneity.

Hypothesis:

- 1) We hypothesized that unbiased proteomic analysis of the influence of different treatments on response to TBI across multiple paradigms might reveal common pathways of repair/regeneration. Such pathways would then become a focus for identification of therapeutic targets of relevance to the heterogeneous human TBI patient population.

5.2 Methods

5.2.1 Study groups

Given the complications that could ensue from the discussion of the multiple different study cohorts under discussion in this chapter, for the sake of simplicity we have adopted the following abbreviations (Table 4).

Study cohort	Abbreviation
Young 5r-mTBI, Nilvadipine (<i>Chapter 3</i>)	5r-mTBI/N
5r-mTBI, Delayed Anatabine (<i>Chapter 4</i>)	5r-mTBI/A
cr-mTBI, Delayed Anatabine (<i>Chapter 4</i>)	cr-mTBI/A
Experimental groups	Abbreviation
Sham-Vehicle	SH-V
TBI-Vehicle	TBI-V
Sham-Treatment	SH-T
TBI-Treatment	TBI-T
Group ratios	Abbreviation
TBI-Vehicle vs Sham-Vehicle	[TBI-V/SH-V]
TBI-Treatment vs Sham-Treatment	[TBI-T/SH-T]
TBI-Treatment vs TBI-Vehicle	[(TBI-T/SH-T)/(TBI-V/SH-V)]

Table 4. Abbreviations of the study cohort and treatment groups.

5.2.2 Tissue extraction and homogenization

Phosphoproteomic analysis was performed using cortex homogenates from the previously described studies with the time points as described in Table 5. Cortex tissue was homogenized using 500 μ l Mammalian Protein Extraction Reagent (MPER) per sample containing 1% EDTA and 1% protease/phosphatase inhibitors cocktail. Samples were sonicated 3 x 10 min and centrifuged at 10,000 rpm at 4°C for 10 min. Supernatants were separated and used for further analysis.

Study cohort	Cortex extraction	Age at death
Young 5r-mTBI Nilvadipine	21 days post last TBI	4 months
5r-mTBI Anatabine	6 months post last TBI	9 months
Cr-mTBI Anatabine	6 months post last TBI	12 months

Table 5. Time points

5.2.3 Sample Preparation

Frozen tissue homogenates were thawed and their protein content was determined using a bicinchoninic acid (BCA) assay. Samples were immediately aliquoted to avoid variation in the protein concentrations due to freeze/thaw cycles. A control sample was created by pooling an aliquot containing 60 μ g of protein from each of the 48 samples into one Eppendorf tube. Next, six 150 μ g aliquots of this control sample were transferred to separate 2 mL Eppendorf tubes. A 10 μ L aliquot of a protein standard mixture was added to each control replicate and 100 mM triethylammonium bicarbonate buffer (TEAB) was added appropriately to adjust the final volume to 100 μ L. For the 5r-mTBI/A and 5r-mTBI/N cohorts, 150 μ g of protein was aliquoted per sample into 2 mL Eppendorf tubes. The protein standard mixture addition and the final volume adjusted were repeated as per the control sample replicates. Due to the limited amount of the samples, 50 μ g of protein per sample of the cr-mTBI/A cohort was aliquoted. Samples were randomized and divided into six batches with one overlapping repeat sample per batch for between batch quality control validation. At this stage all batches were frozen at -20°C.

Further sample processing was performed in batches and was guided by Thermo Scientific's instructions for TMT 10-plex Mass Tag Labeling Kits and Reagents, although not strictly

followed. Each batch consisted of one control replicate, eight true samples and one repeat sample. Processing of one or two batches of samples was started every two days.

Protein reduction and alkylation were accomplished by the addition of 5 μ L of 200 mM tris(2-carboxyethyl) phosphine (TCEP) per sample, incubated for 1 hour at 55°C, followed by 5 μ L of 375 mM iodoacetamide, incubated for 30 min at room temperature in the dark. The protein was then precipitated with 600 μ L of cold acetone, and the process proceeded overnight at -20°C. The next day, samples were pelleted by centrifugation at 8000 x *g* for 15 min at 4°C. The supernatant was aspirated, and pellets were allowed to dry for approximately 20 minutes. The protein was then resuspended in 100 μ L of 50 mM TEAB via a 1-hr incubation in a thermocycler at 37°C followed by a 1.5-hour bath sonication with intermittent vortexing.

5.2.4 Protein Digestion

Protein was digested first with Lys-C, incubated in a thermocycler at 37°C for 1 hour, and then with Trypsin, incubated in the thermocycler at 37°C overnight. Samples originally containing 150 μ g of protein received 3 μ L of (0.25 μ g/ μ L) Lys-C, and those originally containing only 50 μ g of protein received 1 μ L. (0.5 μ g/ μ L) Trypsin was added in the same manner. Samples were then stored at -20°C until ready for peptide labeling.

5.2.5 Peptide Labeling

TMT10plex labeling reagents were brought to room temperature and dissolved in anhydrous isopropanol. For each batch, the combined sample was labeled with TMT-126 and the repeat sample was labeled with TMT-131. Labeling of true samples were randomized. Each sample was combined with one mass-tagging reagent and incubated at room temperature for 1

hr. Subsequently, 8 μ L of 5% hydroxylamine was added to each sample and incubated for 15 minutes to quench the reaction. The total volume for each labeled sample was combined into one new Eppendorf tube and stored at -20°C. Once all batches reached this stage, the six samples were desalted using Pierce C18 spin columns per product instructions. The instructions include an optional wash step for the removal of excess TMT reagent. The samples were then concentrated on a SpeedVac until sample volumes had been reduced to approximately 25 μ L and then stored at -20°C.

5.2.6 Phosphopeptide-enrichment

Phosphopeptide enrichment was performed using TiO_2 beads using steps modified from Huang et al. (Huang et al. 2015). Various acidic acetonitrile solutions were used for the sample buffer, and for peptide binding and washing. Ammonia hydrate solutions were used for the elution phase. Sample buffer was added to the samples and the resulting solution was transferred pipetted into an Eppendorf tube containing conditioned TiO_2 beads. Sample tubes were placed on a shaker for 1 hour to facilitate binding of the phosphopeptides. TiO_2 beads were collected after a brief spin on a tabletop mini-centrifuge and the supernatant representing the unbound peptide fraction was removed and saved. The beads were washed with a three-step procedure. The supernatants were discarded after these steps. Bound analytes, enriched in phosphopeptides, were eluted in two steps with the addition of ammonia hydrate solutions. The supernatants were saved after each elution step and combined. The phosphopeptide enriched fractions were then concentrated on a SpeedVac until volumes had been reduced to 25-50 μ L. Next, an aliquot of 0.1% TFA (aq.) was added to each in preparation for clean-up on Pierce C18 spin columns per product instructions.

5.2.7 LC/MS/MS Analysis

Samples were analyzed on a LC/MS system comprised of an Easy nLC 1000 (Thermo) coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer with a NanoFlex source (Thermo Scientific). Peptides were trapped on an Acclaim PepMap 100 (75 μ m X 20 mm, Thermo Scientific) and desalted. Chromatography was performed on an analytical column (75 μ m X 150 mm, Thermo Scientific) packed with C18, 2 μ m particles using a 115 minute water/acetonitrile reversed-phase gradient method.

The Q Exactive was operated in data-dependent acquisition (DDA) mode. Full scan MS spectra (m/z 400-1800) were acquired in the orbitrap analyzer with a resolving power of 70000 (at m/z 200). The fifteen most intense multiply charged ions ($z \geq 2$) were sequentially isolated with a 1.0 Da isolation width and fragmented in the collision cell by higher-energy collisional dissociation (HCD). Fragment ions were mass analyzed to a 35000 resolving power (at m/z 200).

5.2.8 Peptide and Protein Identification and Quantification

Raw data were processed using Proteome Discoverer software (version 2.1, Thermo Scientific). The MS/MS spectra were searched against a Uniprot mouse protein database (downloaded February 2018) using a target-decoy strategy. Reporter ion intensities were extracted from nonredundant peptide spectral matches (PSMs) and the ratios were determined relative to the combined sample.

5.2.9 Data Analysis

Results were obtained for a total of 12 groups (sham-vehicle, TBI-vehicle, sham-treatment, TBI-treatment) across 3 cohorts (5r-mTBI/nilvadipine, 5r-mTBI/anatabine, cr-mTBI/anatabine). Data were presented as the abundance ratio (fold changes) of protein for each group and were further normalized to the cohort-specific sham-vehicle values. The changes in protein levels were considered significant if the abundance ratio was >1.5 (*upregulated*) or <0.5 (*downregulated*).

The interaction analysis of the dysregulated phosphoproteins was performed using STRING platform (Szklarczyk et al. 2019). Accession numbers were uploaded into an online platform where the background values for species *Mus Musculus* were applied. The platform automatically identifies proteins according to the uploaded accession numbers and shows possible interactions based on the experimental data from existing studies.

Note: The actual MS-based procedures were performed by our MS core staff.

5.3 Results

Overall, 861 phosphoproteins were identified across all cortex extracts from the three studies.

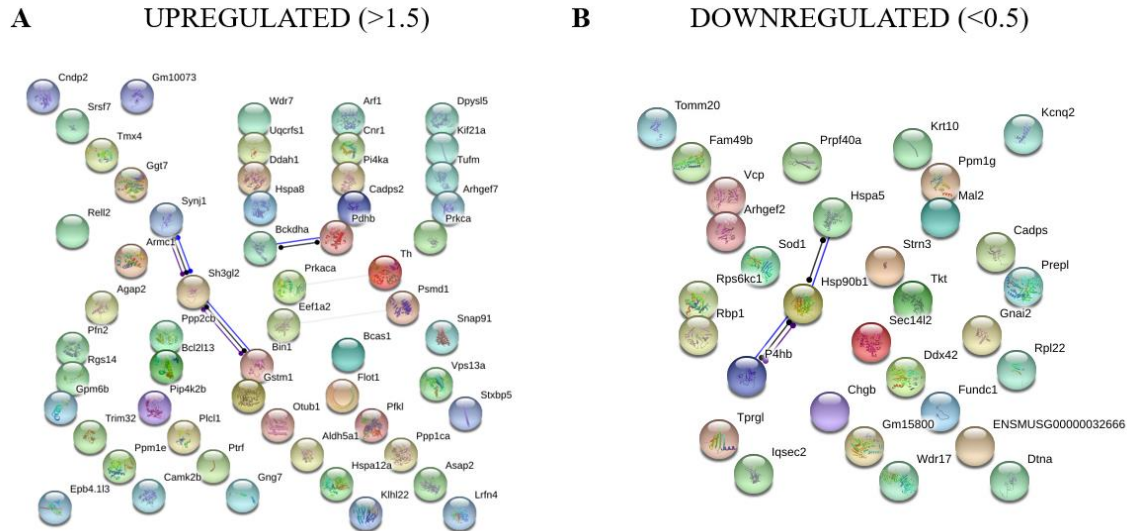
5.3.1 Proteome profiles in different models of r-mTBI

First, TBI response was determined by calculating the fold change of TBI-V to SH-V for each cohort. The lowest number of significantly regulated phosphoproteins in the [TBI-V/SH-V] groups was detected for the cr-mTBI/A: with **69** phosphoproteins *upregulated* (↑) and **33** *downregulated* (↓) (Fig. 28). In the 5r-mTBI/A group, [TBI-V/SH-V] showed **203** (↑) and **52** (↓) phosphoproteins (Fig. 29). In the 5r-mTBI/N, there were **57** (↑) and **447** (↓) phosphoproteins (Fig. 30).

In the (TBI-N/SH-N)/(TBI-V/SH-V) groups, the number of the altered phosphoproteins was oppositely dysregulated compared to cohort-matched TBI-V/SH-V groups: in the cr-mTBI/A - **45** (↑) and **189** (↓) (Fig. 28); in the 5r-mTBI/A group - **88** (↑) and **221** (↓) (Fig. 29); in the 5r-mTBI/N - there were **73** (↑) and **478** (↓) phosphoproteins (Fig. 30).

cr-mTBI/A: (TBI-V/SH-V)

Response to cr-mTBI injury at 3 months post-last injury



cr-mTBI/A: [(TBI-T/SH-T)/(TBI-V/SH-V)]

Influence of delayed anatabine treatment on response to cr-mTBI injury

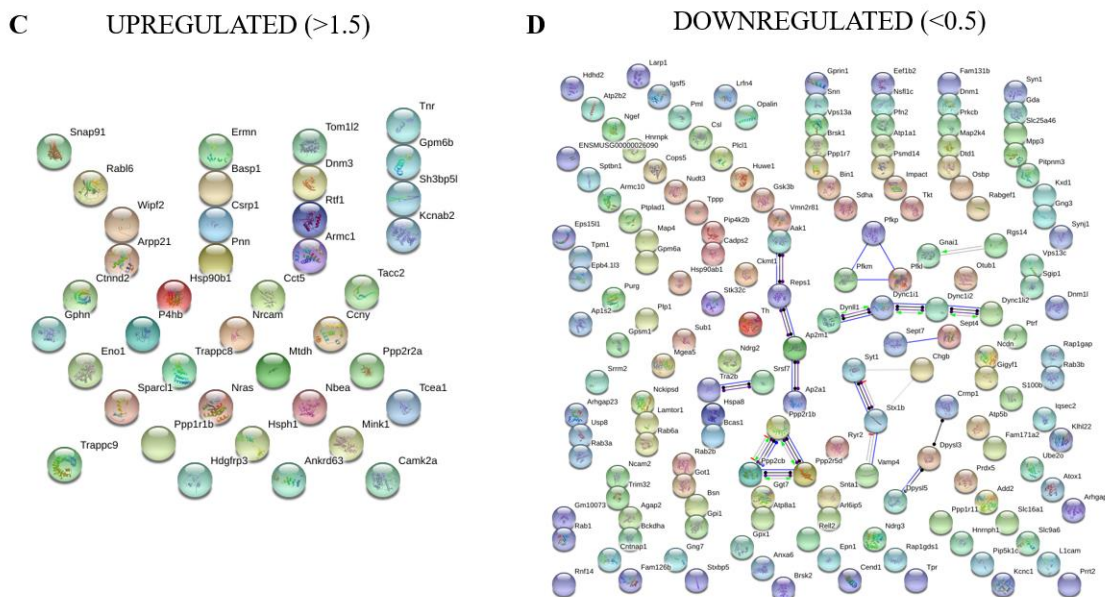
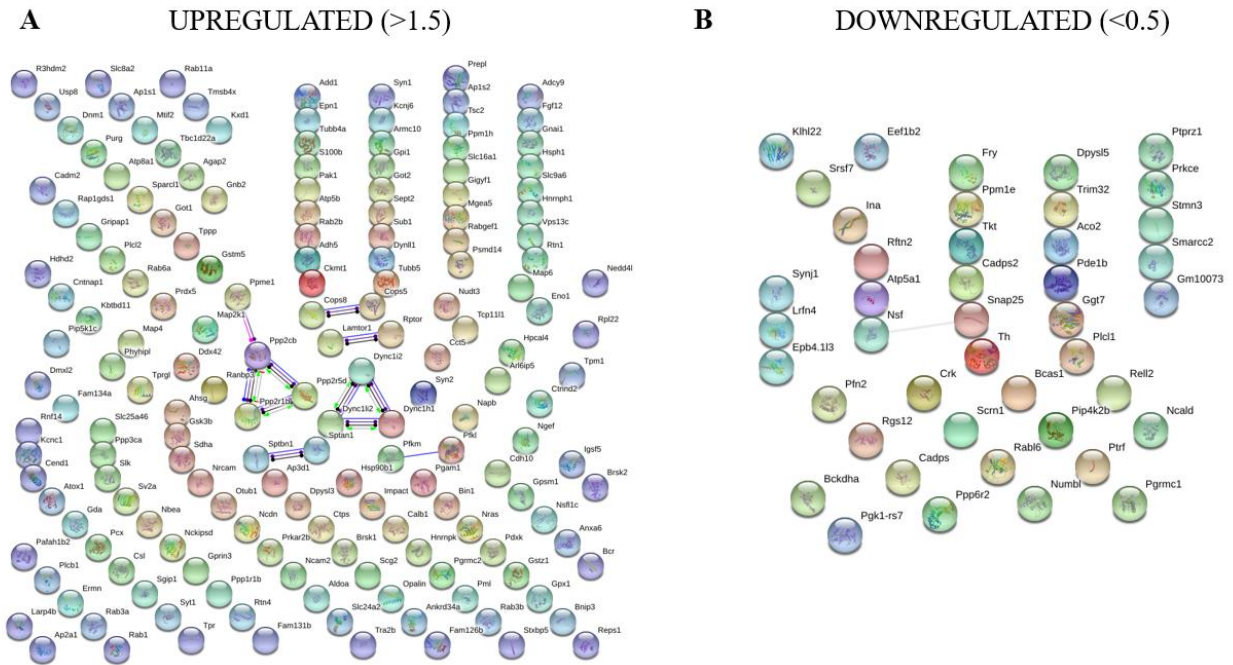


Figure 28. Protein-protein interactions in the cr-mTBI/A cohort

5r-mTBI/A: (TBI-V/SH-V)

Response to 5r-mTBI injury at 3 months post-last injury



5r-mTBI/A: [(TBI-T/SH-T)/(TBI-V/SH-V)]

Influence of delayed anatabine treatment on response to 5r-mTBI injury

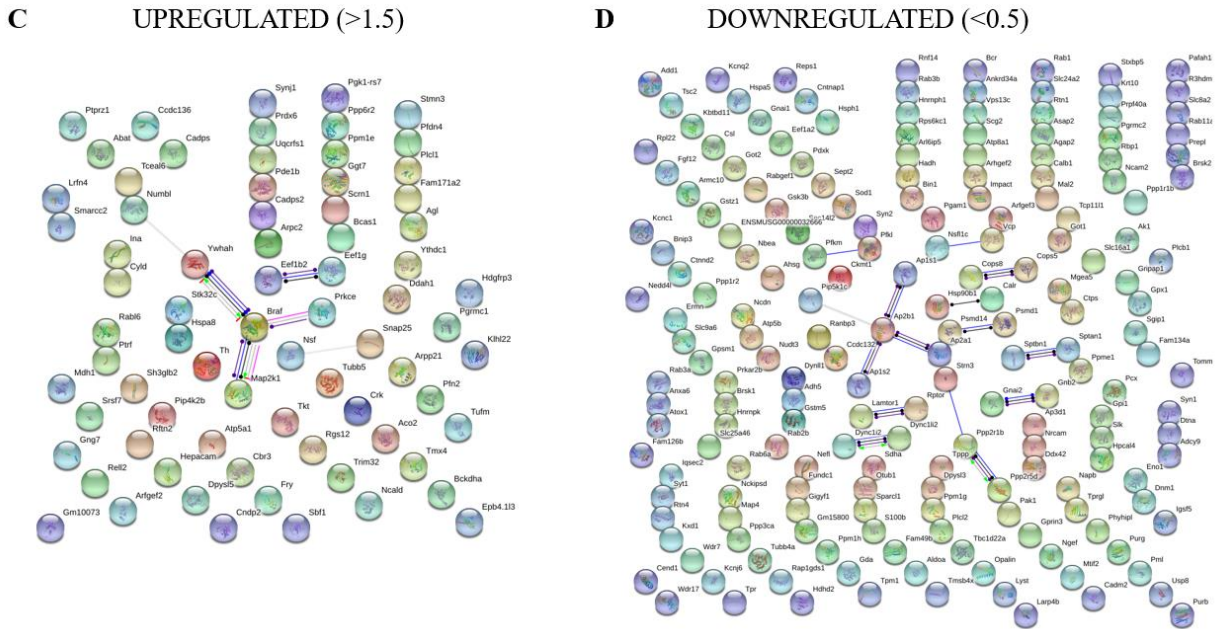
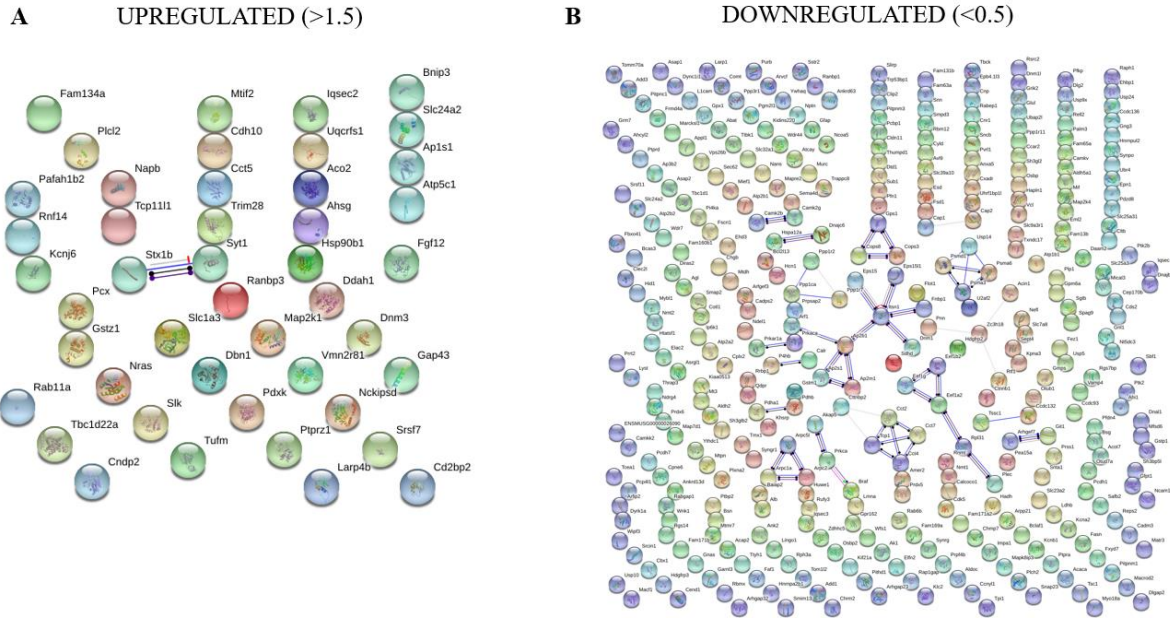


Figure 29. Protein-protein interactions in the 5r-mTBI/A cohort

5r-mTBI/N: (TBI-V/SH-V)

Response to 5r-mTBI injury at 21 days post-last injury



5r-mTBI/N: [(TBI-T/SH-T)/(TBI-V/SH-V)]

Influence of delayed nilvadipine treatment on response to 5r-mTBI injury

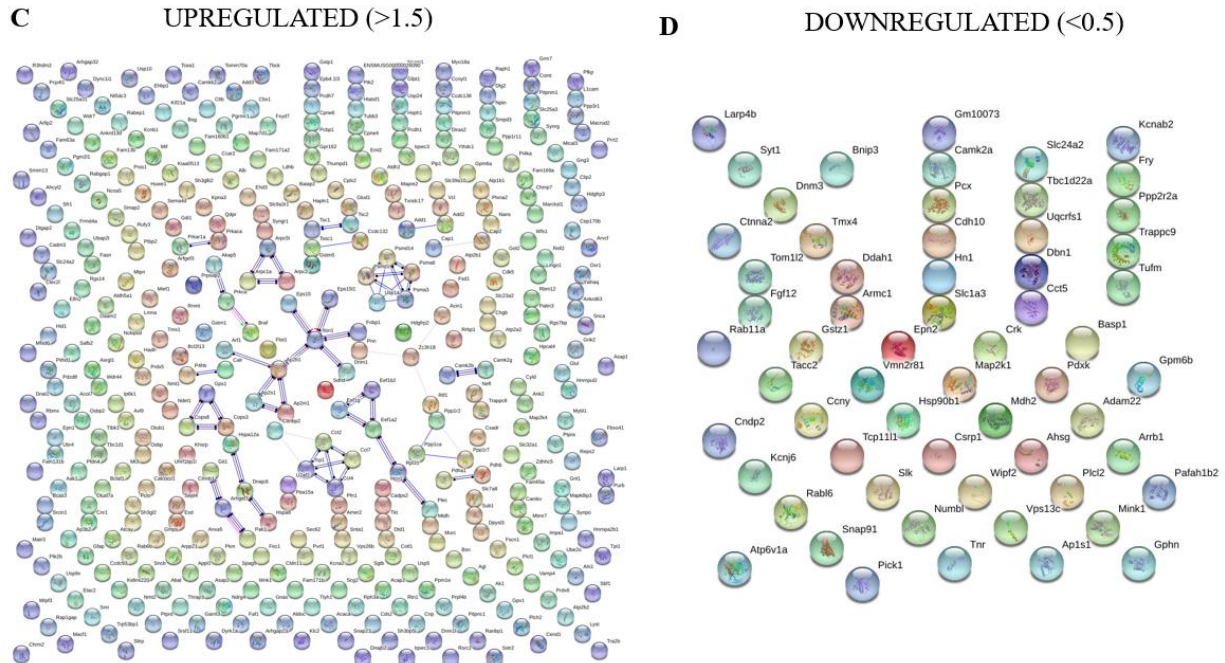


Figure 30. Protein-protein interactions in the 5r-mTBI/N cohort

5.3.2 Phosphoprotein changes across different models

The comparison of the [TBI-V /SH-V] across three different studies revealed only one *upregulated* phosphoprotein (neurofascin (NFASC)) common to response to injury in all three models, and no common *downregulated* phosphoproteins (Fig. 31). Between different pairings of the group the number of common *upregulated* or *downregulated* phosphoproteins was as follows: 5r-mTBI/N & 5r-mTBI/A – **35** (\uparrow), **3** (\downarrow); 5r-mTBI/N & cr-mTBI/A – **9** (\uparrow), **2** (\downarrow); 5r-mTBI/A & cr-mTBI/A – **6** (\uparrow), **2** (\downarrow).

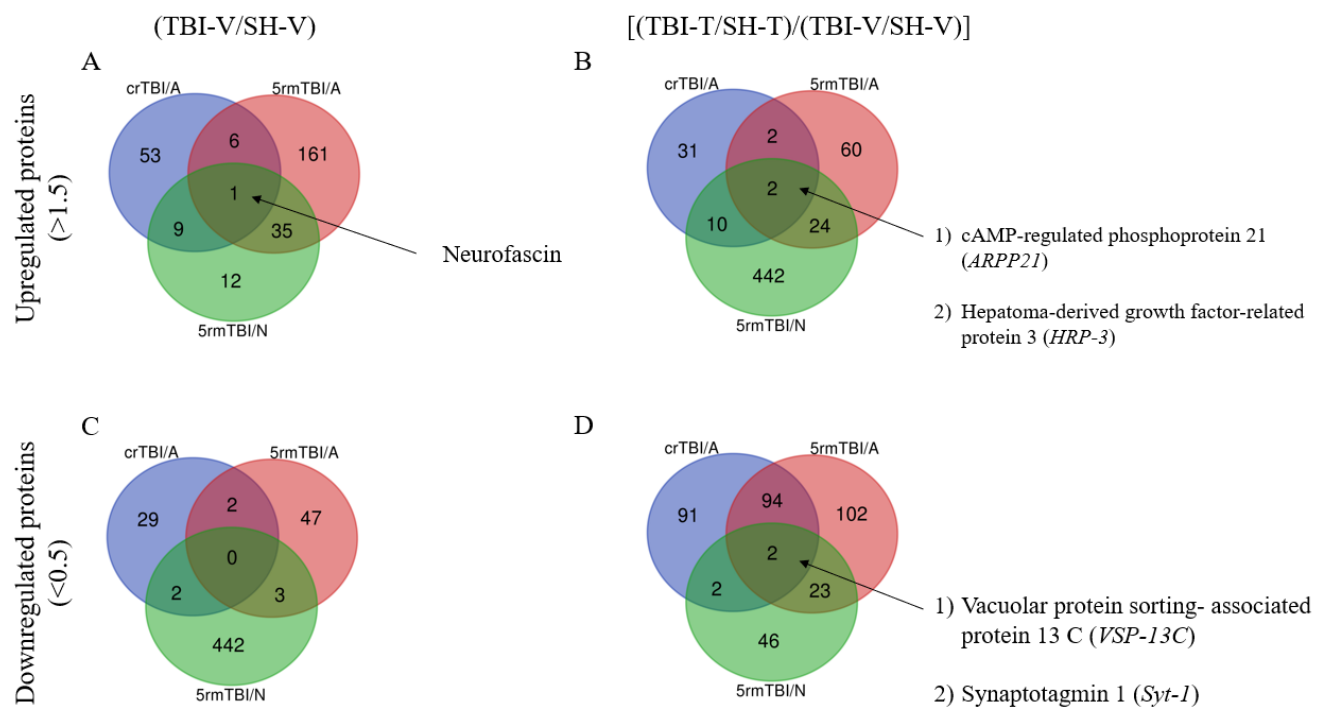


Figure 31. Venn diagrams of the dysregulated phosphoproteins in response to TBI-V (A,C) and TBI-T (B,D).

The comparison of the altered phosphoproteins in terms of the influence of treatment on response to injury [(TBI-T/SH-T)/(TBI-V/SH-V)] between all 3 studies revealed **2** common phosphoproteins that were *upregulated* - cAMP-regulated phosphoprotein 21 (*ARPP21*) and hepatoma-derived growth factor-related protein 3 (*HRP-3*) - and **2** *downregulated* - synaptotagmin 1 (*Syt-1*) and vacuolar protein sorting-associated protein 13C (*VSP13C*). The distribution of the dysregulated phosphoproteins between any two studies showed the maximum overlap to be in the number of *downregulated* phosphoproteins in the two anatabine treatment studies (5r-mTBI/anatabine & cr-mTBI/anatabine) – **94**.

Further analysis of these **94** phosphoproteins, which were *downregulated* in response to treatment, demonstrated that **6** of them were *upregulated* after TBI in both groups (Fig. 32)

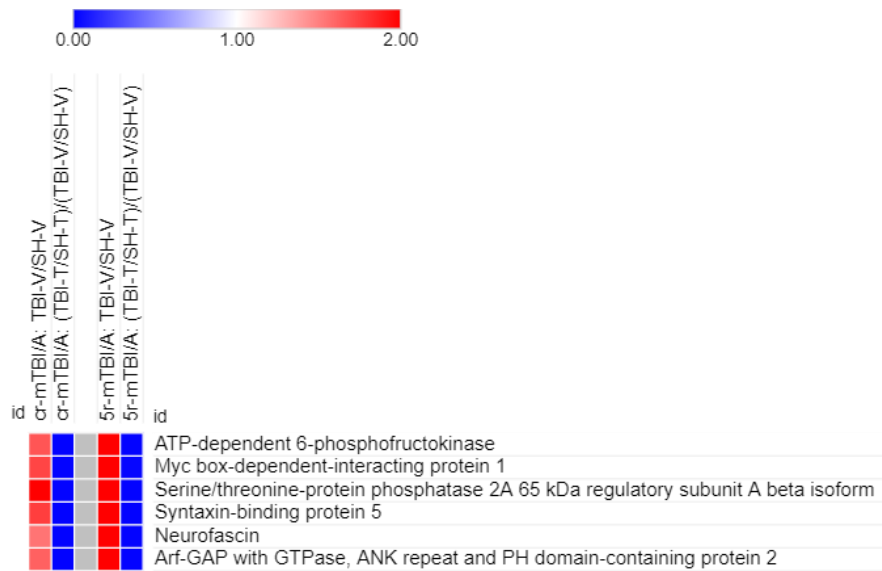


Figure 32. Heatmap of selected phosphoproteins that were *upregulated* in the TBI-V/SH-V and *downregulated* in the (TBI-T/SH-T)/(TBI-V/SH-V) groups in for both 5r-mTBI/A and cr-mTBI/A cohorts.

5r-mTBI/A: [(TBI-T/SH-T)/(TBI-V/SH-V)]; UPREGULATED

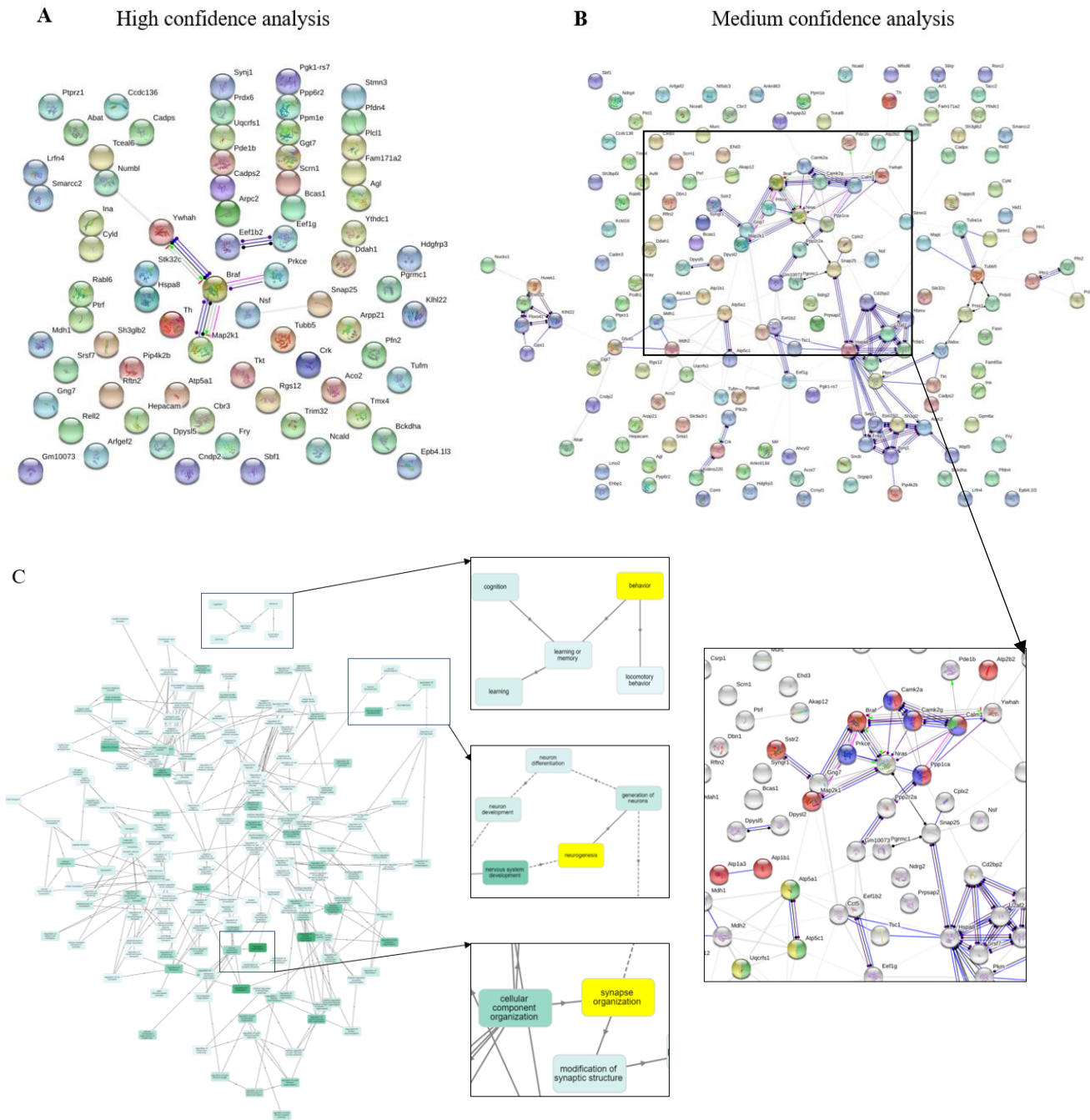


Figure 33. Variability in the analysis outcome demonstrated for the upregulated phosphoproteins in the 5r-mTBI/A [(TBI-T/SH-T)/(TBI-V/SH-V)] cohort. A – previously utilized analysis that applied high confidence interaction score. B – alternative analysis that applied medium confidence interaction score reveals a greater number of protein-protein

interactions. Selected section represents phosphoproteins associated with several TBI-related pathways: **red** – cAMP signaling pathway, **blue** – inflammation, **yellow** – Parkinson's Disease related pathways, **green** – Alzheimer's Disease related pathways. C – pathway analysis demonstrates a large number of altered processes associated with upregulated phosphoproteins. Selected areas show the affected TBI-related processes such as behavior (cognitive and locomotor), neurogenesis, and synaptic organization.

5.4 Discussion

The goal of this chapter was to conduct a preliminary proteomic investigation of response to treatment in our different mouse models, and specifically to identify phosphoproteins that were similarly affected by treatments across the three cohorts. These cohorts differ in the type of drug administered, therapeutic time window, time at first administration, age and injury model. We proposed that phosphoproteins that were similarly altered in response to treatment may represent potential targets that would be common across heterogeneous human TBI populations.

Overall, a total of 861 phosphoproteins were identified. It is important to note that a full interpretation of these data will require the pending Total proteome analysis from the same samples, so that the significance of protein phosphorylation (i.e. the phospho:total) can be assessed.

First, we assessed the effect of TBI on these phosphoproteins for each model. The lowest number of dysregulated phosphoproteins was found in the cr-mTBI/A cohort when compared to 5r-mTBI/A and 5r-mTBI/N. Interestingly, the cr-mTBI/A treated cohort received a greater number of hits when compared to the other groups (24 vs 5 hits) and were significantly older by the end of the study (12 months vs 9 months and 4 months), and might thus have been expected to show a larger number of significantly modulated phosphoproteins. As we discussed in Chapter

2, one of the possible reasons for a lower TBI signal in the aged mice may be a masking effect of age-related pathology. Our proteomic data appear to support this concept, however further analysis is warranted.

Interesting findings were observed when we compared the TBI effect in 5r-mTBI/A and the 5r-mTBI/N groups. Both models used the same injury paradigm (5 hits) administered in 3 months old mice, but the tissue extraction was performed at different time points post injury - 3 weeks (5r-mTBI/N) or 6 months (5r-mTBI/A) – allowing the analysis of temporal proteome changes within the same model. We found that a higher number of phosphoproteins were *downregulated* at 3 weeks post-TBI (**447**) vs 3 months (**52**), whereas *upregulated* phosphoproteins were less prevalent at acute (**57**) versus chronic (**203**) time points. These data suggest very distinct molecular profiles of acute vs chronic phases supporting our expectation that the efficacy of therapeutic targets will likely be dependent on time post-injury. For example, in the chronic but not acute phase, we observed an *upregulation* of several serine/threonine kinases including glycogen synthase kinase beta (*GSK3 β*). *GSK3 β* is involved in tau phosphorylation and amyloid beta production, and was marked as a key element in the AD pathogenesis (Hernandez et al. 2012). Previously, the enzyme's activity was shown to be dysregulated after TBI, while its inhibition was linked to reduced cell death and improved cognitive functions (Dash et al. 2011). Chronic *upregulation* of *GSK3 β* may underly tau phosphorylation found in our 5r-mTBI-V mice at 3 months post-injury (Chapter 4) but not at 3 weeks post-injury (Chapter 3). Such temporal heterogeneity of pathological markers warrants investigation of different therapeutic time windows for potential target candidates.

When we compared the phosphoproteome profile for the 5r-mTBI/N cohort in response to injury or treatment, we found that TBI caused a *downregulation* of **442** unique

phosphoproteins and the same number of unique *upregulated* phosphoproteins in response to nilvadipine. There is a considerable overlap (93%) in the proteins being *downregulated* in response to injury and *upregulated* following treatment, suggesting that the 5r-mTI model triggered an acute decrease in the phosphorylation of a large number of proteins, all of which were restored by nilvadipine. This apparent recovery of the “normal” phenotype may be a feature of the much shorter timeframe of this particular study, and the fact that treatment was administered acutely after injury.

Further, to identify common therapeutic targets across treatments, we found **4** proteins that were either *up-* or *downregulated* in response to all of the treatments in the TBI mice. One of the *upregulated* proteins between all TBI-T groups was cAMP-regulated phosphoprotein 21 (*ARPP21*). The primary function of cAMP signaling is the activation of protein kinase A (PKA) and phosphorylation of transcription factors that are involved in cell survival and the suppression of the expression of pro-inflammatory cytokines (Titus et al. 2013). Phosphorylation of cAMP is regulated by calcium ions, and, due to a significant Ca^{2+} influx after TBI, cAMP is expected to be *upregulated*, as it had been shown in models of stroke and epilepsy (Tanaka 2001). However, multiple studies have demonstrated a *downregulation* of cAMP across different models of TBI (Atkins et al. 2007; Titus et al. 2013). Pre-clinical studies also showed that *downregulation* of cAMP is linked to memory worsening, and treatment with agonists of cAMP restores normal cognitive functions (Titus et al. 2013). An increased amount of phosphorylated ARPP21 was demonstrated in all our models after treatment with either anatabine or nilvadipine. ARPP21 was previously shown to be a positive regulator of dendritic growth, and the knockdown of ARPP21 gene was correlated with a decrease in dendritic complexity (Rehfeld et al. 2018). *Upregulation* of ARPP21 appears to have crucial role in neurorecovery, but whether it can be a

primary target for TBI is still unclear. The second *upregulated* protein was hepatoma-derived growth factor-related protein 3 (*HRP-3*). To our knowledge, there are no studies addressing the effect of TBI on HRP-3. While members of HRP family are expressed in various tissues, HRP-3 is unique to neurons (El-Tahir et al. 2006). A significant role of HRP-3 was demonstrated for neurite growth suggesting its potential role in neurorecovery (El-Tahir et al. 2009).

Surprisingly, TBI-T mice showed a significant decrease in phosphorylated synaptotagmin 1 (*Syt-1*), a calcium-binding protein located in the membranes of the synaptic vesicles which contributes to the docking mechanism required for exocytosis (Wen et al. 2017). Proteolysis of Syt-1 prevents docking of the vesicles to presynaptic membrane affecting mediators release and disrupting synaptic functions. Studies showed that Syt-1 is usually decreased after subarachnoid hemorrhage (Chen et al. 2013) and TBI (M. C. Liu et al. 2006), which may lead to poor neurorecovery and worsened synaptic plasticity. Surprisingly, our study showed that treatment with nilvadipine or anatabine decreased Syt-1 in spite of expected *upregulation*. Again, this may be explained by our current focus on analysis of phosphoproteins. In the case of Syt-1, its phosphorylation is regulated by Ca^{2+} -calmodulin complex (Popoli 1993) which was *downregulated* in some of our models (5r-mTBI/N and 5r-mTBI/A) and may potentially affect the functioning of Syt-1. The second *downregulated* phosphoprotein was vacuolar sorting associated protein 13 C (*VSP13C*). Several studies proposed that mutations in VSP13C gene lead to mitochondrial stress, mitophagy and cognitive impairments associated with the early onset of PD (Lesage et al. 2016; Schormair et al. 2018). However, due to the lack of data on VSP13C in TBI, its role remains unclear.

We then focused on comparison of the influence of anatabine across two different models (5r-mTBI and cr-mTBI). We expected to observe a greater number of overlapping

phosphoproteins by excluding mice treated with nilvadipine. Indeed, we identified a total of **2** *upregulated* and **94** *downregulated* phosphoproteins common to the response to treatment in both models, which included S100 β , GSK3 β , and protein phosphatases, all of which were originally *upregulated* in at least one of the TBI-V groups. As we mentioned earlier, GSK3 β has been linked to tau phosphorylation and amyloid accumulation in the AD models (Hernandez et al. 2012). Additional studies also showed a TBI-induced *upregulated* of GSK3 β which was prevented by treatment with the enzyme inhibitors (Shim and Stutzmann 2016). The protein S100 β is widely used as a marker of astrocytic activation, hence a decreased phosphorylation of this protein may be an indicator of reduced inflammation in our models (Braun et al. 2017). We also revealed **6** phosphoproteins from this dataset that were *upregulated* after TBI in the absence of treatment. They included serine/threonine phosphatase, synaptic proteins (Neurofascin, syntaxin-binding protein) and proteins associated with plasma membrane functioning.

We would like to acknowledge that, owing to the limited time, our study at this time has analyzed only phosphoproteins and not total proteins, which are required for a complete picture of protein changes. This limitation will be addressed in our future studies where we plan to map protein phosphorylation against total changes in the same tissue we used here. We also plan to conduct a more detailed analysis of the identified peptides by modeling the interaction of known proteins with compounds outside of our dataset. Moreover, applying less stringent criteria during the interaction analysis may reveal a greater number of protein-protein connections that have been missed in the current data analysis. Overall, this study presents valuable data demonstrating high heterogeneity of TBI and possible therapeutic targets that may be effective across different models.

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Chapter 6 - Summary

The overarching goal of this dissertation was to address several of the current gaps in the TBI preclinical field by building on our previous work on characterization and development of preclinical models of r-mTBI to investigate the effects of different potential treatments and include clinically relevant administration paradigms. In the course of this work we developed the following aims: 1) To replicate heterogeneity of human concussions by using two mouse models of r-mTBI; 2) To test two potential TBI therapeutics - nilvadipine and anatabine - that have demonstrated safety in humans; 3) To address the long-term consequences of mTBI by exploring delayed and chronic treatment paradigms; and 4) To investigate common therapeutic responses across these multiple approaches in order to identify viable, translational targets for further investigation.

6.1 Addressing Aim 1

To address **Aim 1** and to mimic the clinical TBI heterogeneity, we used two injury paradigms - 5r-mTBI and cr-mTBI - that use the same mTBI injury but involve 5 versus 24 hits, respectively, and thus mimic different injury frequency and chronicity. We found that both models replicably induced chronic astrogliosis and tau phosphorylation and increased nuclear NFkB activation. We also demonstrated TBI-dependent cognitive impairment with both models. However, only the cr-mTBI model was associated with significant motor deficits, whereas only the 5r-mTBI triggered disinhibition behavior. We have previously observed motor deficits with the 5r-mTBI model acutely after injury (B. Mouzon et al. 2012), but these have not persisted. The persisting motor deterioration in the cr-mTBI mice thus appears to be associated with a greater number of hits and may also be related to the older age of mice at the time of testing.

The inconsistent presentation with disinhibition is difficult to interpret at this time and will require further investigation. Despite these neurobehavioral variations, our study demonstrated that, assessed at 6 months post-last injury, 5 mild hits over a short period of time (9 days) can trigger equally severe long-term pathological outcomes and memory deficits as 24 hits over 3 months.

The high heterogeneity of human concussions has been suggested as a possible reason behind the failures of all the drugs introduced into clinical development for TBI (DeWitt et al., 2018; Mohamadpour et al. 2019). As we find strong evidence for neuroinflammation in all of our preclinical models, and to the best of our knowledge this is a feature of *all* laboratory models and human patient samples (Pearn et al. 2017; V. E. Johnson et al. 2013; Pavlovic et al. 2019; McKee et al. 2014), we exposed both models of r-mTBI to the same anti-inflammatory treatment, anatabine. We observed that anatabine attenuated TBI pathology equally in both models but had inconsistent effects on memory deficits. In the cr-mTBI, anatabine improved spatial memory, but this effect was not detected in the 5r-mTBI mice. Intriguingly, cr-mTBI mice were 3 months older than 5r-mTBI at the time of behavior assessment (11-12 months vs 8-9 months old, respectively), and 12 months, but not before, is the age when cognitive impairments appear in healthy hTau mice (M. Polydoro et al. 2009). Hence, memory impairments in the cr-mTBI might have been triggered by both TBI and old age, whereas behavioral problems in the 5r-mTBI mice were driven solely by TBI. It is surprising to see that anatabine was more effective in mice with, supposedly, greater extent of cognitive deficits, but, perhaps, the level of severity of the pathological environment is crucial for a drug to reach its therapeutic peak. Interestingly, in our previous work with anatabine treatment in wild type mice subjected to the 5r-mTBI, delayed treatment with anatabine at 9 months post-last injury also did not result in significant cognitive

improvement, despite a positive effect on inflammation (S. Ferguson et al. 2016). However, in that study the mice were 21 months old at the time of assessment and we deduced that the advanced age of the mice had confounded detection of any improvement in cognitive performance. Interpretation of these findings is difficult at the moment and requires further analysis before drawing final conclusions regarding the effect of anatabine on cognition. This highlights the need to understand both the treatment time window and the length of treatment needed to produce beneficial results. The discrepant data in terms of functional/behavioral outcomes, particularly when considering the same treatment across different injury paradigms, might certainly be considered to reflect the complexities of translation to human clinical trials and supports the need for much more thorough preclinical characterization of any proposed treatments.

Implications:

We suggest that preclinical testing of potential therapeutics should be performed in different models of mTBI to account for heterogeneity and to provide more accurate conclusions regarding the likely effects of treatment in human patients.

6.2 Addressing Aim 2

To address **Aim 2**, we explored 2 treatments – nilvadipine and anatabine. Ideally, we intend to investigate nilvadipine in the delayed treatment 5r-mTBI and cr-mTBI paradigms, to provide a direct comparison with our anatabine data. For now, nilvadipine was administered acutely for the duration of 21 days post-TBI whereas anatabine was tested as a delayed treatment starting at 3 months post last injury. These drugs have different mechanisms of action: nilvadipine is syk

inhibitor and anatabine is a nAChR agonist. Nevertheless, both drugs attenuated TBI-induced pathology including neuroinflammation and tau phosphorylation and caused model-specific improvements of behavior performance.

Nilvadipine has been associated with robust positive effects in animal models of AD and with clinical benefits for patients with early stage AD in a phase III randomized, controlled trial (Abdullah et al. 2019; Lawlor et al., 2018). Post mortem studies indicate that there is a biological link between TBI and AD pathology (e.g. inflammation, tauopathy, more rarely - amyloidopathy), suggesting that treatments that are effective in AD might also be effective in TBI. In the AD models, nilvadipine attenuates neuroinflammation, tau phosphorylation and amyloid accumulation (Paris, Ait-Ghezala, et al. 2014). Although we do not see amyloid pathology in our r-mTBI model, considering post-injury neuroinflammation and tau as malleable responses common between TBI and AD, our positive results in AD thereby support the use of nilvadipine as potential therapeutic agent for the chronic effects of TBI. Indeed, we showed that nilvadipine exhibited similar therapeutic effect on TBI-induced inflammation and tau phosphorylation in young and aged mice.

A nAChR agonist anatabine has been previously shown to decrease inflammation in the models of AD, EAE, and LPS-induced inflammation (Paris, Beaulieu-Abdelahad, Abdullah, et al. 2013; Verma et al. 2015), and given a featured role of neuroinflammation in TBI pathology, anatabine might be effective in TBI. Together, our previous (S. Ferguson et al. 2016) and current findings support the ability of anatabine to decrease TBI-induced inflammation but they also indicate its role in reducing tau phosphorylation and improving motor and cognitive functions (in the cr-mTBI mice).

Implications:

Both nilvadipine and anatabine demonstrate efficacy in our preclinical models of r-mTBI. Given that both have previously demonstrated safe use in humans, their further investigation as potential treatments for mTBI is warranted.

6.3 Addressing Aim 3

A likely scenario for treatment of r-mTBI patients is that they will not begin treatment until some time has elapsed since the injuries, once clinical symptomatology emerges. We therefore sought to address **Aim 3** by investigating the effects of delayed treatment in our models, and for this we used the anti-inflammatory anatabine, administered at 3 months post-last injury. Anatabine attenuated TBI-induced pathological outcomes in both models (cr-mTBI and 5r-mTBI) and improved behavior functions (in the cr-mTBI mice). These data confirm the possibility of a chronic therapeutic time window for TBI and warrants further investigation. Our approach highlights the importance of broadening the preclinical approaches to drug discovery to address the problems that have become apparent with clinical translation.

Implications:

Our data suggest that delayed treatment with an anti-inflammatory drug was an effective approach with which to target chronic outcomes of r-mTBI. More preclinical studies should implement a delayed treatment approach to mimic relevant human conditions as it may reveal novel properties of the existing treatment candidates and introduce new therapeutics.

6.4 Addressing Aim 4

To address **Aim 4** and to identify common therapeutic targets across different models, we conducted a phosphoproteomic analysis of cortex extracts from the mice treated with nilvadipine or anatabine (Chapter 3-4). Despite a highly heterogeneous phosphoproteome profile between the groups, we identified 4 proteins that were dysregulated in all the models in response to treatment. These proteins were associated with synaptic functions, neuronal growth and dendritic complexity, and may indicate treatment-induced increases in synaptic plasticity. Multiple studies have linked dendritic branching and the number of synapses with cognitive performance indicating its importance in neurorecovery. However, whether they represent an effective target requires further investigation. We have not yet conducted the total proteome analyses for these samples which are needed in order to fully understand the protein responses to TBI and to treatment in these models. Overall, we have data to suggest a complex protein response to various models of TBI, which underlie pathological heterogeneity and may contribute to the failures of clinical trials.

Implications:

The demonstrated heterogeneity of the phosphoproteomic profiles in our r-mTBI models underlines the likelihood that different therapeutics will have efficacy in different injury paradigms, and at different times of administration post injury.

6.5 Future directions

Throughout the studies we presented in this dissertation, we collected an extensive number of samples that represent a very heterogeneous dataset of r-mTBI conditions from which

we evaluated cognitive and pathological outcomes in response to injury and treatment. At the final stage we attempted to combine these samples for the MS-based proteomics analysis to identify molecular targets that are similarly dysregulated across the models and may represent possible therapeutic targets. We plan to proceed with the investigation of proteomic profiling of the existing data by conducting a total proteome analysis and mapping it against our current dataset. Once we are able to analyze the disturbed phosphoproteome in relation to the total proteome we plan to conduct a more thorough investigation of all the phosphoproteins identified to be significantly regulated, and cluster them according to their signaling pathways. Moreover, our next step will involve the analysis of the phosphorylated sites of the detected proteins followed by the identification of predicted protein interactions outside our dataset.

6.6 Conclusion

Overall our data support the fact that the therapeutic window for intervention in the negative sequelae of r-mTBI may be quite large, which is very encouraging for the patient population who may not seek treatment until many months or even years after they sustained the injuries. However, our data also clearly demonstrate similarities and differences across different injury paradigms (even with the same injury impact), addressing, at a very small scale, the problem of the heterogeneity of the human TBI patient population. Our data continue to support the contention that much more rigorous testing of potential therapeutics in different models of TBI is required before drawing strong conclusions regarding the efficacy of the treatment, and prior to attempting their clinical translation.

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